UNIVERSIDADE ESTADUAL DE CAMPINAS
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LÍCIA CARLA DA SILVA COSTA

COMPREENSÃO DA DEPRESSÃO GERIÁTRICA ATRAVÉS DE BIOLOGIA DE SISTEMAS A PARTIR DA ANÁLISE PROTEÔMICA DE PLASMA SANGUÍNEO

UNDERSTANDING LATE-LIFE DEPRESSION EMPLOYING SYSTEM BIOLOGY-BASED PROTEOMICS

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Orientador: PROF. DR. DANIEL MARTINS DE SOUZA

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“Somewhere, something incredible is waiting to be known.”
— Carl Sagan

“Let us think the unthinkable, let us do the undoable, let us prepare to grapple with the ineffable itself, and see if we may not eff it after all.”
— Douglas Adams

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RESUMO

O distúrbio chamado depressão geriátrica, ou, em inglês, late-life depression (LLD) corresponde a episódios depressivos que ocorrem por volta dos 60 anos de idade ou mais, mas possui mecanismos fisiopatológicos do LLD distintos da depressão maior. Assim, uma visão integrada dos processos biológicos e das vias bioquímicas alteradas em pacientes com LLD pode contribuir para o diagnóstico, acompanhamento da resposta ao tratamento ou mesmo a escolha de medicamentos.

Neste trabalho, empregamos a proteômica com base na espectrometria de massas, com o objetivo de identificar potenciais proteínas associadas ao LLD e vias bioquímicas relacionadas, o que conduzirá à exploração dos processos patogênicos que levam ao distúrbio. Inicialmente realizamos a depleção da fração de proteínas mais abundantes do plasma, para melhor investigarmos a fração com proteínas menos abundante. Em seguida, utilizamos o método de espectrometria UDMS^{E}, que nos provê uma cobertura melhor do proteoma complexo do plasma.

Obtivemos, então, 96 proteínas que sugerem uma expressão diferencial significativamente diferente. Essas proteínas estão relacionadas ao sistema imunológico, em especial à resposta inflamatória e à cascata de regulação do sistema complemento. Encontramos, também, 6 proteínas associadas ao aumento da severidade da depressão, que participam de diversas vias bioquímicas.

Assim, este trabalho apresenta descobertas que confirmam a LLD como um distúrbio de fisiopatologia heterogênea, com diferentes vias contribuindo para diferentes fenótipos. A relação desses conjuntos de vias bioquímicas afetadas com diferentes sintomas apresentados pelos pacientes, pode fornecer pistas para investigação de novos alvos farmacológicos e também para novas abordagens terapêuticas. Para isso, estudos envolvendo uma coorte maior são necessários para um delineamento molecular mais preciso das diversas condições que coexistem na LLD.

ABSTRACT

Late-life depression (LLD) corresponds to depressive episodes that occur around the age of 60 or more, yet differing in pathophysiological mechanisms when compared to major depression. For this reason, an integrated view of biological processes and altered biochemical pathways in patients with LLD may contribute to an improved diagnosis, more concise follow-up of treatment response or even help in the selection of medication.

Here, we employ proteomics based on mass spectrometry aiming to identifying potential proteins associated with LLD and its related biochemical pathways, which will lead to the exploration of pathogenic processes that lead to this disorder. First, we performed a depletion of the most abundant protein fraction of the plasma, to better investigate the fraction with less abundant proteins. Then, we use a UDMS^{E} method, which provides us with a better coverage of the complex plasma proteome.

We then obtained 96 proteins though in silico analysis whose expression levels were significantly altered in LLD patient samples. These proteins are related to the immune system, in particular to the regulating cascade of the complementary system. We also found 6 proteins associated with the increased severity of depression, which participate in various biochemical pathways.

In summary, these findings confirm that LLD is a disorder of heterogeneous pathophysiology, with different pathways contributing to different phenotypes. The relationship of these biochemical pathways with different symptoms displayed by patients may provide clues for the investigation of new pharmacological targets and also for new therapeutic approaches. For this, however, further studies involving a larger cohort are necessary for a more precise molecular portrayal of the various conditions that coexist in the LLD.

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

LISTA DE ABREVIAÇÕES

2D-PAGE	Eletroforese bidimensional em gel de poliacrilamida
BDNF	Fator neurotrófico derivado do cérebro
DDA	Método de aquisição dependente de dados
DIA	Método de aquisição independente de dados
DSM 5	Manual diagnóstico e estatístico dos transtornos mentais 5
HDMSE	Espectrometria de massas de alta definição
HDRS	Escala de Hamilton para Depressão
LC	Cromatografia líquida
LC-MS	Cromatografia líquida associada à espectrometria de massas
LLD	Late-life depression ou depressão geriátrica
MDD	Depressão maior
MSE	Espectrometria de massas com aumento progressivo na energia de colisão
SASP	Proteínas secretadas por diferentes fibroblastos senescentes
UDMSE	Espectrometria de massas de ultra definição

SUMÁRIO

1. INTRODUÇÃO

1.1. Apresentação

A organização desta dissertação foi planejada de forma a exibir o trabalho desenvolvido pela autora durante aproximadamente os dois anos que antecederam à publicação deste material. O primeiro capítulo traz uma revisão bibliográfica reunindo os trabalhos mais recentes relacionados a possíveis assinaturas moleculares da depressão maior; este capítulo foi publicado como parte integrante do livro *Reviews on Biomarker Studies in Psychiatric and Neurodegenerative Disorders*, que compõe a série de livros chamada *Advances in Experimental Medicine and Biology*, publicada pela Springer Nature em 2019, doi: 10.1007/978-3-030-05542-4_10.

O segundo capítulo traz a discussão sobre como as proteínas que podem compor potenciais painéis de biomarcadores são perdidas durante o processo de depleção por cromatografia de imunoafinidade. Essa discussão foi explorada em poucos artigos que antecederam esta publicação. Embora a existência da interação entre essas proteínas seja bem conhecida, este artigo tem o intuito de aumentar o corpo de conhecimento sobre a identidade dessas proteínas. O artigo foi publicado na revista *Journal of Separation Science Plus* em setembro de 2019, doi: 10.1002/sscp.201900057.

O terceiro capítulo é um protocolo detalhado para análise do proteoma de plasma sanguíneo a partir do método de UDMS^{E}. Este método foi o mesmo empregado nas análises que compõe o quarto e último capítulo. Este manuscrito está em fase final de preparação, e será publicado como parte integrante do livro *Shotgun Proteomics: Methods and Protocols* que faz parte da série de livros *Methods in Molecular Biology*, publicados pela Springer Nature.

O quarto e último capítulo apresenta as análises do proteoma do plasma sanguíneo de pacientes com depressão geriátrica, e traz os resultados da investigação da expressão diferencial destes pacientes em relação a indivíduos idosos na ausência de distúrbios psiquiátricos. Este manuscrito encontra-se em fase final de preparação para submissão em periódico indexado de circulação internacional.

A dissertação é finalizada com as conclusões obtidas a partir desses estudos; as limitações identificadas são apontadas brevemente, e as perspectivas futuras são delineadas.

1.2. A depressão maior e sua relação com a depressão geriátrica

“Na depressão, a falta de significado de cada empreendimento e de cada emoção, a falta de significado da própria vida, se tornam evidentes. O único sentimento que resta nesse estado despido de amor é a insignificância” (Solomon, 2000, p.7). Neste trecho do livro “O Demônio do Meio-dia”, o autor traz uma visão pessoal, possivelmente, de uma das características da depressão, a anedonia (SOLOMON, 2014). A depressão é caracterizada por humor deprimido (sentimento de tristeza, vazio e desesperança), anedonia (redução do interesse em atividades que antes eram prazerosas ao indivíduo), perda ou ganho significativo de peso, alterações no sono, perturbações psicomotoras, fadiga e ideações suicidas (AMERICAN PSYCHIATRIC ASSOCIATION, 2013). Também, conforme o Manual Diagnóstico e Estatístico dos Transtornos Mentais (DSM 5), o diagnóstico de depressão ocorre quando cinco ou mais desses sintomas estão presentes por, pelo menos, duas semanas, incluindo humor deprimido e anedonia.

Estimativas feitas pela Organização Mundial de Saúde em 2015 mostram que ela afeta 4,4% das pessoas do planeta, sendo mais comum em mulheres (5,1%) do que em homens (3,6%) (WORLD HEALTH ORGANIZATION, 2017). Também, sabe-se que depressão é uma doença complexa e está associada a fatores ambientais - principalmente a exposição ao estresse, fatores genéticos/epigenéticos, e traços de personalidade (LOPIZZO et al., 2015; POST, 1992; VIALOU et al., 2013). A depressão é uma das principais comorbidades psiquiátricas que surgem durante doenças graves, sendo comum sua coexistência com a doença de Alzheimer, possuindo sobreposição dos sintomas resultantes do declínio cognitivo nesta doença (LYKETSOS; OLIN, 2002b). Sintomas de depressão também pode estar associados a padrões regulatórios de expressão gênica que levam a uma maior ocorrência de Alzheimer. Por exemplo, prejuízos cognitivos são comuns em adultos com depressão, assim como sintomas depressivos são frequentes em idosos diagnosticados com Alzheimer. As duas doenças parecem compartilhar vias bioquímicas, como, por exemplo, de controle imuno-inflamatório e suporte neurotrófico, que levam a prejuízos neurológicos (MENDES-SILVA et al., 2016).

De acordo com a Organização Mundial de Saúde, a população mais afetada pela depressão é composta por pessoas entre 55 a 74 anos, sendo, portanto, uma condição comum e incapacitante em idosos. Assim, cerca de 6,5% da população

nesta faixa etária é atingida pela doença, sendo que mais de 7,5% de mulheres neste grupo é afetada pela depressão, contra 5,5% dos homens com mesma idade (WORLD HEALTH ORGANIZATION, 2017). Considerando a intensificação do uso dos sistemas de saúde e medicamentos por idosos atingidos pela depressão, o maior fardo para familiares e cuidadores, dentre muitos outros aspectos envolvidos na doença, a incidência de depressão nessa faixa etária implica na diminuição da qualidade de vida tanto dos idosos quanto dos familiares, assim como um problema econômico e social crescente (ZIVIN; WHARTON; ROSTANT, 2013).

A progressão do envelhecimento cerebral ao longo da vida envolve alguns genes que alteram sua expressão durante o envelhecimento, sendo que a suscetibilidade às doenças mentais dependerá de variações genéticas, contexto molecular de cada indivíduo e de fatores ambientais (SIBILLE, 2013). A expressão "depressão geriátrica" se refere frequentemente aos episódios depressivos que ocorrem por volta de 60 anos de idade ou mais tarde (sendo que o primeiro episódio pode ocorrer ainda na vida adulta - early-onset depression-, ou durante o envelhecimento - late-onset depression). Pode ser descrita como uma síndrome neuropsiquiátrica heterogênea e complexa, desencadeada por outra doença grave, por comorbidades, ou ser resultado da utilização de medicamentos (AZIZ; STEFFENS, 2013)

1.3. Hipóteses associadas à patofisiologia da depressão maior e depressão geriátrica

A depressão maior pode ter sua origem parcialmente explicada por algumas hipóteses diferentes. A hipótese monoaminérgica, que parte do pressuposto que a depressão é um distúrbio no sistema monoaminérgico (redução da disponibilidade de serotonina ou noradrenalina), cunhada em meados da década de 60 tornou-se uma das hipóteses mais populares sobre a etiologia da depressão (LÓPEZ-MUÑOZ; ALAMO, 2009; SCHILDKRAUT, 1965). Entretanto, nos anos subsequentes, a observação de que apenas o sistema monoaminérgico não era suficiente para explicar a resposta ao tratamento continuado da depressão, assim como a resposta a outros tratamentos - como eletroconvulsoterapia - que não interagem com neurônios monoaminérgicos, contribui para o nascimento da chamada hipótese glutamatérgica, que tomou forma em meados dos anos 90. Assim, esta

hipótese se baseia em alterações nas funções cerebrais, envolvidas no controle de humor, devido aos níveis excessivos de sinapses glutamatérgicas (PAUL; SKOLNICK, 2003; SANACORA; TRECCANI; POPOLI, 2012).

Outros mecanismos foram propostos para explicar a fisiopatologia da depressão. Evidências de desequilíbrio no sistema imunológico (como processos inflamatórios) e interações de proteínas sinalizadoras (como citocinas) com o sistema nervoso central, gerando alterações comportamentais, deram origem à hipótese neuroimunológica (MAES, 1995; SCHIEPERS; WICHERS; MAES, 2005). A esta hipótese relacionam-se as duas outras, as hipóteses neurotrófica e de neuroplasticidade, pois a ativação do sistema neuroimune leva à redução de fatores neurotróficos, como o fator neurotrófico derivado do cérebro (BDNF). Essa redução de fatores neurotróficos, além de reduzir a neuroplasticidade, também dificulta a neurogênese e a formação de novas sinapses, mudanças compatíveis com a observação de prejuízo nos processos cognitivos relacionados à doença (HAYLEY, 2014; PITTENGER; DUMAN, 2008; TONG et al., 2008).

Uma das propostas para o surgimento da depressão geriátrica é chamada de hipótese vascular: distúrbios vasculares cerebrais e seus fatores de risco, e/ou crescente acúmulo de lesões vasculares cerebrais silenciosas com consequente aumento de substância branca podem mediar diversos mecanismos que colaboram para o surgimento ou permanência da depressão geriátrica, como consequência de de danos nos circuitos cerebrais (ALEXOPOULOS, 1997). Entretanto, esta hipótese ainda não é amplamente aceita porque idosos podem ser igualmente vulneráveis a diversas doenças, incluindo problemas cardiovasculares e outras comorbidades, além dos efeitos colaterais dos tratamentos utilizados nessas doenças, que aumentam o risco de depressão geriátrica. Assim, a depressão vascular pode ser considerada como um subtipo de depressão geriátrica, embora mais estudos sejam necessários para o estabelecimento de seus mecanismos, devido à pouca compreensão acerca das mudanças bioquímicas e estruturais no cérebro que podem associar a hipótese da depressão vascular à depressão geriátrica (AIZENSTEIN et al., 2016).

A investigação da expressão diferencial de proteínas periféricas, através de imunoensaios em plasma sanguíneo de pacientes idosos, levou a associação entre a depressão geriátrica e processos inflamatórios e redução de suporte neurotrófico, além de marcadores de proteostase e detecção de nutrientes (DINIZ et al., 2016). Também, alguns desses mesmos marcadores mostram que a desregulação

homeostática presente em pacientes com depressão geriátrica pode acelerar os processos de envelhecimento, que aumentarão o risco de Alzheimer e demência (DINIZ et al., 2013, 2015). Outro estudo importante correlacionou maior índice SASP (conjunto de proteínas secretadas por diferentes fibroblastos senescentes, responsáveis por induzir o envelhecimento de células próximas), em pacientes com depressão, e pode dar pistas sobre os mecanismos moleculares que culminam em depressão geriátrica (COPPÉ et al., 2008; DINIZ et al., 2017). Entretanto, ainda são poucos os estudos com o propósito de investigar os mecanismos moleculares associados à LLD e, talvez assim, obter uma assinatura molecular consistente da doença.

1.4. Identificação de assinaturas moleculares da LLD através da proteômica

Considerando que os mecanismos patofisiológicos da depressão geriátrica são distintos da depressão maior, uma visão integrada dos processos biológicos e vias bioquímicas alteradas durante o distúrbio podem contribuir para o diagnóstico, acompanhamento da resposta ao tratamento ou mesmo escolha do medicamento. Neste aspecto, a proteômica baseada em espectrometria de massas é um conjunto de ferramentas com potencialidade para atender esta demanda, que ganhou notoriedade nos últimos anos por sua capacidade de resolver a complexidade de amostras de proteínas, como o plasma sanguíneo, gerando dados sobre a sequência, modificações, quantidade, estrutura e contexto biológico das proteínas a partir de fragmentos de peptídeos (AEBERSOLD; MANN, 2016).

O termo proteoma (WILKINS et al., 1996) pode ser definido como um conjunto de proteínas expressas por uma célula, tecido ou organismo, em dado momento, sob determinadas condições. A proteômica, a ciência que estuda o proteoma, surgiu na era pós-genômica como um conjunto de ferramentas robustas para investigação de doenças multifatoriais e/ou com contribuição relevante do ambiente; dentre elas, as doenças psiquiátricas. Esse conjunto de ferramentas bioquímicas trabalham juntas para, dentre outras aplicações, desvendar a complexidade de doenças como a esquizofrenia, distúrbio bipolar e depressão (NASCIMENTO et al., 2016).

Ferramentas proteômicas também são empregadas para a descoberta de biomarcadores. Estes podem compor assinaturas moleculares que podem vir a ser utilizadas como indicadoras para diagnóstico, monitoramento da doença ou marcadores de resposta bem sucedida a um tratamento. Muitos desses marcadores estão disponíveis na circulação sanguínea e a utilização desse tipo de amostra é favorável ao estudo das doenças psiquiátricas, tendo em vista que sua obtenção é razoavelmente pouco invasiva.

1.4.1. O plasma sanguíneo como fonte de potenciais biomarcadores

O plasma sanguíneo, devido sua facilidade de acesso, é uma fonte interessante de biomarcadores proteicos. Deve-se considerar, entretanto, que cerca de 20 proteínas bem caracterizadas compõe 99% do peso do plasma sanguíneo, e estas proteínas, devido à sua abundância e à resolução dos instrumentos de análise, tendem a ofuscar a visualização de potenciais biomarcadores contidos na fração de baixa abundância, presentes em 1% do plasma (JAROS et al., 2013a). .

Assim, alguns pesquisadores optam por submeter as amostras a um processo de depleção, que é a remoção da fração de alta abundância, para investigação de proteínas biomarcadoras no plasma sanguíneo (GARCIA et al., 2017; JAROS et al., 2013b). A cromatografia por imunoafinidade é considerada o estado-da-arte para separação dessas proteínas, onde a fase estacionária da coluna cromatográfica é composta por anticorpos imobilizados com afinidade por proteínas de alta abundância do plasma. A depleção baseada em anticorpos policlonais mostrou-se altamente específica e com ótima reprodutibilidade, porquanto é capaz de reter proteínas de alta abundância quando submetidas a determinadas condições de carregamento, enquanto proteínas de baixa abundância podem ser eluídas através da coluna para posterior análise proteômica (ZOLOTARJOVA et al., 2008).

1.4.2. Cromatografia líquida de alta eficiência associada à espectrometria de massas

Amostras biológicas são amostras complexas que podem conter milhares de proteínas. Nestas amostras, a presença de proteínas com propriedades físico-químicas semelhantes pode dificultar a separação e posterior quantificação e

identificação dessas proteínas. Diversas técnicas de separação, para aumentar a cobertura de identificação dessas moléculas, podem ser utilizadas, separadamente ou em conjunto com outros métodos.

A cromatografia líquida (LC) associada à espectrometria de massas (LC-MS) tem sido amplamente empregada em experimentos convencionais de proteômica em larga escala, com o intuito de aumentar a cobertura do proteoma investigado e melhorar a sensibilidade analítica, em especial de proteínas de baixa abundância (Link et al. 1997; Eng et al. 1994). A técnica de identificação de proteínas empregando LC-MS foi inicialmente denominada *shotgun proteomics*, ao viabilizar a identificação, em larga escala, de uma mistura de proteínas (LINK et al., 1999a). Quando a amostra de proteína é digerida com enzimas proteolíticas conhecidas, antes de LC-MS, a técnica é chamada de *bottom-up*, pois a informação sobre a proteína é recomposta a partir de fragmentos de peptídeos identificados, de forma análoga ao sequenciamento genômico (YATES, 1998; ZHANG et al., 2013). Mais recentemente, a técnica denominada *top down* também tem sido empregada, ao analisar proteínas intactas em larga escala.

Bottom up shotgun proteomics começou a ser empregada efetivamente nos anos 2000 em substituição a eletroforese bidimensional em gel de poliacrilamida (2D-PAGE), que foi a base das origens da proteômica. *Shotgun proteomics*, em princípio, supera algumas das limitações de 2D-PAGE como a necessidade de análise de cada ponto (ou *spot*) presente no gel, a dificuldade em separar proteínas de baixa abundância, proteínas integrais de membrana e com pontos isoelétricos ou massas moleculares extremos (Washburn et al. 2001).

Desta forma, um experimento clássico de *bottom up shotgun proteomics* realiza a digestão da amostra com enzimas proteolíticas adequadas, e os peptídeos, de acordo com suas características físico-químicas, são separados através de um cromatógrafo líquido acoplado diretamente a um ionizador. Em seguida, os peptídeos ionizados são conduzidos por um gás neutro para o interior de uma câmara utilizando um campo elétrico, onde serão, em seguida, fragmentados, gerando íons precursores e íons produtos; a razão massa/carga desses peptídeos, agora ionizados, é então registrada no detector (ZHANG et al., 2013). A fragmentação é realizada, portanto, na fase gasosa e os espectros de íons fragmentados são adquiridos. Neste momento, são geradas as informações sobre identidade e a quantidade relativa de peptídeos. Então, através da inferência de proteínas e usando ferramentas computacionais, os

peptídeos identificados são re-atribuídos às suas proteínas originais. A vantagem potencial da proteômica *shotgun* é que ela pode ser adotada geralmente para todos os tipos de proteínas, uma vez que os peptídeos são mais facilmente fracionados em cromatografia líquida, ionizados e fragmentados, do que as proteínas intactas (AEBERSOLD; MANN, 2016; ZHANG et al., 2013).

1.4.3. Aquisição de dados

Ferramentas proteômicas são especialmente úteis para investigação amostras complexas, como o plasma sanguíneo humano, onde as concentrações das proteínas podem variar em mais de dez ordens de magnitude. A complexidade dessas amostras é facilmente explorada através do método de aquisição independente de dados (DIA). Essa abordagem corresponde à coleta de dados de fragmentação multiplexada, consistindo na obtenção de dados espectrais usando os sinais de peptídeos intactos e também o sinal dos peptídeos fragmentados, sem nenhuma seleção prévia dos peptídeos de amostragem (AEBERSOLD; MANN, 2016; CHAPMAN; GOODLETT; MASSELON, 2014).

Em um método DIA chamado MS^E, o quadrupolo é usado para guiar todos os íons para a célula de colisão. Em MS^E, o espectrômetro de massas alterna estados de alta e baixa energia rapidamente. As massas dos peptídeos intactos (aqui considerados íons) são medidos em baixa energia (nível MS1), enquanto que a alta energia promove a fragmentação dos mesmos íons peptídicos (nível MS2). Em seguida, ferramentas de bioinformática são utilizadas para combinar íons precursores e fragmentados, utilizando características como tempo de retenção, razão massa/carga e outros eventuais atributos físico-químicos (LI et al., 2009a).

A adição de outras dimensões de separação, e, consequentemente, um aumento nas identificações dos peptídeos, são características de métodos de fragmentação derivadas do método MS^E. Uma dimensão extra de separação pode ser obtida através do emprego da mobilidade iônica dos íons precursores; isto é, a separação dos íons de acordo com sua forma e carga utilizando as diferentes taxas de movimentação desses íons através da câmara e um gás de amortecimento (VESSECCHI et al., 2011). Assim, à medida que, devido a sua conformação e tamanho, os íons interagem entre si vão perdendo energia cinética, uma separação adicional é promovida. Este método é chamado de espectrometria de massas de alta

definição ($HDMS^E$)(GEROMANOS et al., 2009, 2012). O método $HDMS^E$ utiliza uma energia de colisão que aumenta linearmente durante a fase de colisão de alta energia, e, por isso, alguns íons precursores podem permanecer intactos. Assim, outra dimensão de separação pode ser gerada com o emprego de gradientes não-lineares de energias de colisão em amostras de referência. Este método é chamado de espectrometria de massas de ultra definição ($UDMS^E$), e ele tem sido empregado para aumentar a cobertura de identificação em proteomas complexos, permitindo um controle mais refinado das energias de colisão durante a fase de colisão de alta energia (DISTLER et al., 2014; SOUZA; GUEST; MARTINS-DE-SOUZA, 2017).

Em relação ao método de aquisição dependente de dados (DDA) - método que permite adquirir espectros de fragmentação dos íons mais intensos, dentro de critérios pré-definidos de seleção de íons -, o método DIA traz vantagens como o aumento de identificações de peptídeos precursores em análises exploratórias e em experimentos livres de marcadores. Ainda, para amostras complexas, o método DIA tem a vantagem de diminuir a perda de dados resultante da não-fragmentação de precursores, em especial de precursores resultantes de íons menos intensos originários de peptídeos pouco abundantes, em relação ao método DDA (MICHALSKI; COX; MANN, 2011). Entretanto, existem algumas limitações. Há um aumento nos resultados falso-positivos devido à redução na precisão de massa e sobreposição de espectros, dada a equivalência de massa de diferentes peptídeos (HU; NOBLE; WOLF-YADLIN, 2016; LI et al., 2009b; WIENER et al., 2004). Por isso, a identidade dos peptídeos e proteínas geralmente necessitava de validação através de algum outro método que não espectrometria de massas (PHAN; QUO; WANG, 2006). No entanto, essas limitações estão sendo superadas pelo desenvolvimento de novas tecnologias relacionadas.

1.4.4. Identificação e quantificação de proteínas

O grande volume de dados gerado por essas técnicas proteômicas depende de algoritmos e tratamentos estatísticos para prosseguimento da busca por informações relevantes sobre o objeto investigado. Neste caso, softwares de análise de espectros de ionização se inserem neste cenário como instrumento essencial para identificação e quantificação de proteínas, através de programas contendo algoritmos precisos para avaliar o tempo de retenção, intensidade dos espectros gerados pelos

íons, e relação massa/carga dos íons precursores ou produtos (LI et al., 2009c; TSOU et al., 2016).

Em geral, a análise computacional dos dados brutos realiza alguns passos principais para identificação e quantificação de proteínas. Primeiro, realiza-se a extração dos sinais dos peptídeos originados da separação por cromatografia e representação de cada característica através de relações massa/carga, carga, tempo de retenção de valores de intensidade. Esse passo irá ainda realizar a correção da linha de base, aperfeiçoamento do sinal e detecção dos picos. Em seguida, o alinhamento dos picos é realizado, para procurar correspondência dos tempos de retenção ao longo dos experimentos, de forma que as características das mesmas moléculas possam ser agrupadas(ZHANG et al., 2010). Depois do agrupamento dessas características, o espectro de fragmentação é comparado com um banco de dados, utilizando parâmetros como o tipo de enzima proteolítica, o número de clivagens perdidas durante a digestão e possíveis alterações químicas intrínsecas ao tipo de digestão realizada (HELM et al., 2014).Após identificação dos peptídeos através da comparação dos espectros experimentais com espectros teóricos contra um banco de dados de sequência de proteínas, obtém-se uma lista de sequência de proteínas que correspondem à melhor combinação dentro dos parâmetros fornecidos pelo usuário (revisado em (EDWARDS, 2017). A quantificação das proteínas identificadas em amostras biológicas complexas, tanto no método $HDMS^E$ quanto em $UDMS^E$, pode ser feita através da quantificação relativa das intensidades dos picos dos peptídeos precursores (*MS1-based*) livre de marcadores (*label-free*)(GROSSMANN et al., 2010; SILVA et al., 2006). Este método de quantificação se baseia na relação entre a média dos sinais espectrométricos dos três peptídeos (Hi-3) mais intensos de determinada proteína e sua concentração na amostra (SILVA et al., 2006). Assim, após a identificação dos peptídeos e das proteínas, a abundância de cada peptídeo pode ser calculada a partir de todos os íons correspondentes a estes peptídeos. A organização classificatória das abundâncias dos peptídeos seleciona os grupos de peptídeos de maior confiança, considerando a precisão do alinhamento (SILVA et al., 2006; VÄLIKANGAS; SUOMI; ELO, 2018).

1.5. Análise de biologia de sistemas

A interpretação biológica de dados gerados por técnicas como proteômica *shotgun* pode ser facilitada por uma descrição estruturada de informações biológicas já conhecidas e algoritmos que permitam que esses dados sejam classificados. Plataformas para integração de dados de diversas ciências ômicas abrigam ferramentas que podem adicionar camadas de informações relevantes para a interpretação desses dados (SHANNON et al., 2003; ZHOU et al., 2019). O propósito da biologia de sistemas é investigar condições fisiológicas normais e patológicas e as relações que regem as redes regulatórias e vias sinalizadoras, em células, tecidos, órgãos, até o organismo inteiro, com o propósito de entender o comportamento de sistemas biológicos. Essas análises permitem a geração de modelos de doenças, investigação de mecanismos relacionados à patofisiologia de doenças multifatoriais, previsão de resposta à medicação e a integração de ciências ômicas (revisado em (RAI; RAJ; VARADWAJ, 2018). Ainda, modelamento matemático através de aprendizado de máquina e inteligência artificial provê um caminho para geração de hipóteses e análises *in silico* de biologia de sistemas (BUTCHER; BERG; KUNKEL, 2004; HUNTER; BORG, 2003).

Considerando as ferramentas já disponíveis para investigação de dados proteômicos, a anotação funcional de genes presentes em um conjunto de dados pode ser investigada contra um conjunto de genes de referência, e ensaios estatísticos podem testar a significância de processos biológicos, funções moleculares e localizações de proteínas representativas daquele conjunto de dados. Informações visuais podem ajudar a mostrar quais termos (representados por nós gráficos) são mais significativos e quantos genes estão envolvidos naquela categoria (número representado pela área de cada nó), assim como o tamanho das arestas pode informar quantos genes se sobrepõe naqueles conjuntos conectados (ASHBURNER et al., 2000; MAERE; HEYMANS; KUIPER, 2005).

Também é possível obter informações a nível de sistemas, com o propósito de obter respostas sobre diferentes interações químicas, vias bioquímicas afetadas ou possíveis alvos farmacológicos, para citar alguns exemplos (JASSAL et al., 2020). Informações como enriquecimento de vias, rede de interação de proteínas, e meta-análise de múltiplas listas de resultados podem ser realizadas através de plataformas ortogonais sincronizadas a banco de dados, com o desafio de superar a redundância que possivelmente pode acontecer entre termos ontológicos, quando se reúne vários

bancos de dados diferentes numa mesma plataforma (SZKLARCZYK et al., 2019; ZHOU et al., 2019).

Em suma, através da proteômica baseada em espectrometria de massas, e empregando modelos computacionais para investigação de vias, e análises estatísticas para identificação de potenciais assinaturas moleculares, pode ser possível encontrar pistas proteômicas sobre a LLD no plasma sanguíneo de idosos com depressão, sendo possível correlacionar um painel de proteínas com o estado atual de um grupo de pacientes. Além disso, proteínas diferencialmente expressas e enriquecimento de vias podem dar indicações sobre as cascatas moleculares que levam idosos a apresentarem depressão.

2. JUSTIFICATIVA

A depressão geriátrica é uma doença debilitante e uma comorbidade comum com diversas doenças graves, como a doença de Alzheimer, demência senil, dentre outras doenças. A depressão geriátrica atinge cerca de 6,5% da população de idosos no mundo, representando um problema econômico e social que tende a aumentar com o crescimento e envelhecimento da população.

Assim, a investigação de alterações em proteínas no plasma sanguíneo, associadas à depressão geriátrica, com o propósito de aumentar a compreensão dos mecanismos moleculares envolvidos na patogênese deste distúrbio, pode contribuir para a compreensão da doença. Estes dados podem ser úteis, no futuro, para outros trabalhos que visam a investigação de alvos farmacológicos e eventual desenvolvimento de testes bioquímicos que auxiliem o diagnóstico clínico, e para colaborar com a classificação de diferentes perfis clínicos associados à LLD.

3. OBJETIVOS

O propósito deste trabalho é explorar, através de análise de biologia de sistemas, o perfil proteômico relativo à uma possível assinatura molecular relacionada à depressão em pessoas idosas e identificar proteínas que podem estar correlacionadas com o aumento na pontuação na escala HDRS. Também, este trabalho tem o objetivo de identificar de vias bioquímicas relacionadas à doença, o que permitirá a investigação dos processos patogênicos que levam ao distúrbio e poderá revelar novos alvos terapêuticos. Adicionalmente, vias bioquímicas que podem estar envolvidas em diferentes perfis de pacientes foram investigadas, considerando características clínicas como a presença de prejuízo cognitivo ou depressão recorrente.

4. CAPÍTULO 1

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Proteomic Markers for Depression

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Running title: proteomic markers for depression

Abstract

Major Depression Disorder is a multifactorial disease, with molecular mechanisms not fully understand. A breakthrough could be reached with a panel of diagnostic biomarkers, which could be helpful to stratify patients and guide physicians to a better therapeutic choice, reducing the time between diagnostic and remission. This review brings the most recent works in proteomic biomarkers and highlights several potential proteins that could compose a panel of biomarkers to diagnostic and response to medication. These proteins are related to immune, inflammatory and coagulatory systems, and may also be linked to energy metabolism, oxidative stress, cell communication, and oligodendrogenesis.

Key words major depression disorder, mass spectrometry, antidepressants, drug response

1 General Overview

With 322 million people (4.4% of the population) affected worldwide according to the World Health Organization, major depressive disorder (MDD) is a long lasting and recurrent disorder and one of the leading causes of disability in the western

world, with a lifetime prevalence at almost 15% of the diagnosed patients(BROMET et al., 2011). Difficulties in social and occupational function, suicidal thoughts and decline in physical health may occur in 10-30% of MDD patients who do not respond to treatment, possibly due to the syndrome's heterogeneity, which can make it difficult to diagnose(AL-HARBI, 2012; LAMERS et al., 2013; SOUERY et al., 1999). The boundaries of depressive disorders, whether they can be considered only symptoms or true syndromes, are unclear as their symptoms are sometimes varying and even opposing, such as weight loss or weight gain, insomnia or hypersomnia, and seem to have no established mechanism (BELMAKER; AGAM, 2008; PARIS, 2014; STRIEGEL-MOORE, 2011). Aside from this, the delayed response observed with antidepressants can hinder early observation of good or bad outcomes(DURIC; DUMAN, 2013; FAVA; DAVIDSON, 1996; STASSEN; ANGST; DELINI-STULA, 1997).

MDD is a multifactorial disorder(LOPIZZO et al., 2015; POST, 1992; VIALOU et al., 2013) with several neurobiological hypotheses. The monoaminergic hypothesis, coined in the mid-1960s, postulate that disturbances in the levels of monoamines (serotonin, noradrenaline, and dopamine) are responsible for depression(LÓPEZ-MUÑOZ; ALAMO, 2009; SCHILDKRAUT, 1965). However, the monoaminergic hypothesis was not sufficient to explain all changes observed in depression. In this regard, symptoms can be managed with electroconvulsive therapy or pharmacological manipulations of glutamatergic system(PAUL; SKOLNICK, 2003; SANACORA; TRECCANI; POPOLI, 2012). Despite neurotransmitters, studies have shown the involvement of the immune system in the pathophysiology of depression(MAES, 1995; SCHIEPERS; WICHERS; MAES, 2005). The activation of the neuroimmune system leads to reduction of neurotrophic factors such as the brain-derived neurotrophic factor (BDNF), hampering the neurogenesis, changes that are compatible with the observation of impairment in the cognitive processes related to the disease (HAYLEY, 2014; PITTENGER; DUMAN, 2008; TONG et al., 2008).

Successful treatment of depression is also challenged by its various subtypes, with different neurobiological, biochemical, genetic and anatomical characteristics. The molecular mechanisms of these subtypes are still poorly understood(OSTERGAARD; JENSEN; BECH, 2011; RUSH, 2007; WANG; INSEL, 2010). Still, gene polymorphisms are only considered a risk factor for depression, not a way to diagnose the disease, and few possible candidate single-nucleotide polymorphisms (SNPs) for MDD were considered replicable(BOSKER et al., 2011;

CONVERGE CONSORTIUM, 2015). The concept of endophenotypes in psychiatry reaffirms that the heterogeneity of symptoms of psychiatric illnesses such as MDD is the result of a complex network of interactions between genes, proteins, circuits of cells, and also between individuals and their experiences(GOTTESMAN; GOULD, 2003). In order to establish a relationship between genes and clinical phenotypes, endophytic characterization has allowed some insights related to the mechanisms of MDD, through proteomic, transcriptomic, neuroanatomical, neurological, behavioral and cognitive measurements, which must be inheritable and correlated with the disease, as well as measurable between affected and unaffected individuals, among other criteria(GLAHN et al., 2014; GOLDSTEIN; KLEIN, 2014; HASLER et al., 2004). The construction of endophenotypes can be favored through the establishment of proteomic biomarkers when influenced by genetic factors, although not every biomarker is considered an endophenotype(LENZENWEGER, 2013).

A PubMed search on depression biomarkers reveals to what degree different biomarker approaches are highlighted in this field. In the last 5 years, the use of proteomic approaches in the study of psychiatric disorders has grown considerably, which has led to the identification of a wide array of differentially expressed proteins of which some could be potential biomarkers. Proteomics methods can be performed in various ways, such as with a high-throughput discovery set up or targeted quantitation, as the study of proteins can be performed both individually or in combination with others. Frequently, techniques are based on size characterization or antibody/aptamer binding(GADAD et al., 2018). In this review we analyze different approaches, in terms of scientific merit, for the discovery of biomarkers for MDD.

2 Biomarker characterization

Biomarkers are measurable and evaluable characteristics capable of indicating a disease, a normal biological process or the treatment response(BIOMARKERS DEFINITIONS WORKING GROUP., 2001). Diagnostic biomarkers can help stratify patients with depression, predictive biomarkers can assess response or remission, and moderators can determine the likelihood of response or remission to a particular treatment(GADAD et al., 2018; STRAWBRIDGE; YOUNG; CLEARE, 2017). Furthermore, biomarkers can be useful to identify new molecular targets, aiming to improve the development of new drugs(MACALUSO; PRESKORN, 2012). However, a large data of proteomic biomarkers has been

proposed for MDD without being implemented in the clinic because of lack of sensitivity or specificity, or because standardization norms do not exist or are not widely accepted(JENTSCH et al., 2015; STRAWBRIDGE; YOUNG; CLEARE, 2017). Thus, it is necessary to compose a panel with several biomarkers, so that one set of proteins can display the changes in different biological mechanisms (Fig. 1).

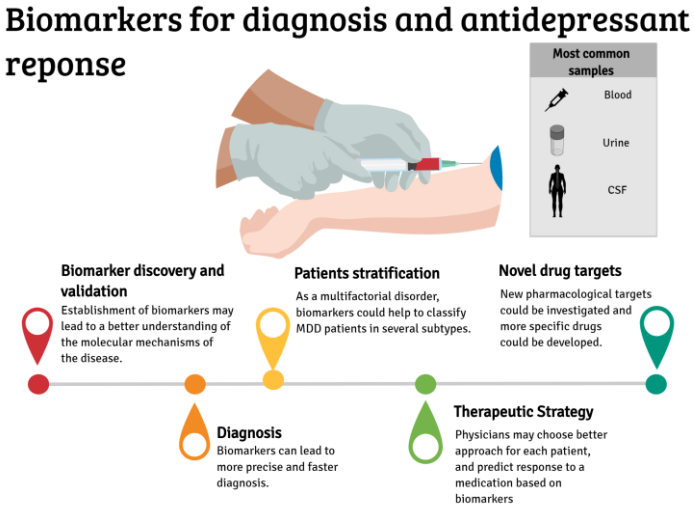

Figure 1 Establishment of biomarkers to MDD can promote a breakthrough in therapeutic strategies

Considering that both diagnostic biomarkers and response biomarkers should serve the ultimate purpose of reducing the overall time between diagnosis and successful treatment, the type of sample to be used for proteomic analysis should be easily obtained and must reflect the health status of the organism(LIM; DICKHERBER; COMPTON, 2011). Therefore, although it is possible to obtain proteomic biomarkers of disease and response in materials such as tissues, cells or through image analysis, for a patient being treated in a clinical setting, samples such as saliva, urine and blood are more suitable for this end(ELLIOTT; PEAKMAN; UK BIOBANK, 2008). Cerebrospinal fluid or fibroblasts could be obtained under more restrictive criteria, as the collection of this body fluid occurs in a relatively invasive manner(SU et al., 2016).

Plasma is one of the most complex human samples with a dynamic range of proteins exceeding 10 orders of magnitude(ANDERSON; ANDERSON, 2002b), and containing proteomic sets of other tissues. It is also an abundant and easily obtained material. Its disadvantages for proteomic analysis include the presence of a large amount of albumin and heterogeneous glycoproteins, which make it difficult to observe other proteins(ANDERSON; LEIGH ANDERSON; ANDERSON, 2003; URBAS et al.,

2009). Plasma is composed of proteins secreted by tissues that act through the bloodstream or are the result of cellular damage; immunoglobulins; local or long-distance receptor ligands, such as hormones and cytokines; temporary passengers, such as lysosomal proteins; and aberrant secretions and proteins foreign to the organism originated from infections(ANDERSON; ANDERSON, 2002b; PUTNAM, 2012). Serum is a proteomic solution resulted from blood clotting, and because of the process of proteolysis, which may alter some proteins, one may prefer plasma to serum(JAMBUNATHAN; GALANDE, 2014).

Other samples and models have been employed for the purpose of delineating the molecular mechanisms of depression, such as animal models, which can help to establish a trustworthy panel of biomarkers, and post-mortem tissue, which can be of limited because lifestyle and treatment of the patients(MARTINS-DE-SOUZA et al., 2009; “Remodeling of axo-spinous synapses in the pathophysiology and treatment of depression”, 2013).

3 Proteomics findings

With the purpose of elucidating the most interesting discoveries involving proteomic biomarkers related to depression, this text initially addresses the studies carried out in the last five years with the purpose of unveiling mechanisms and forming a panel of diagnostic biomarkers. Afterwards, some possible insights into the biochemical mechanisms of MDD made possible by the use of an animal model will be discussed and discoveries made through trials involving the response to treatments will be presented. The articles chosen for discussion were experimental works in patient or animal models that have MDD as theme and have been published in English in the last five years. The keywords used were "depression", "biomarkers" and "proteomics". Pubmed and Scopus were the databases investigated. The main results of this research are summarized in Table 1.

Table 1 Overview of recent potential biomarkers for MDD. Bold genes are recurrent potential biomarkers among the papers surveyed

Gene Name	**Characteristics of sample**	**Biological process**
CP, EN-RAGE, FTH1, HPR, **IL-1**, IL-16,**MIF**, F3, **TNC**	Blood from drug-naive patients	Inflammatory process
APOB, APOD, **CP, GC**, HRNR, PFN1	Blood from drug-naive patients	Immune and inflammatory processes/ lipid metabolism
APOB, CP, GC	Blood from drug-naive patients	Immune and inflammatory processes
CRP, **ITIH4**, **SAA1**, ANGPTL3	Blood from drug-naive patients	Immune and inflammatory processes
ASS1	Urine from drug-naive patients	Inflammatory process /urea cycle
CPE	Post-mortem pituitary tissues from BD and MDD patients	Carboxypeptidase activity
RBP-4, TTR	Blood from BD and MDD patients	Retinoid metabolic process
END, B2RAN2	Blood from BD and MDD patients	Artery morphogenesis/ inflammatory process
CRP, **SAA1**, FX, PCI, TF, FVII, FV, TFPI, APC, F1+2	Blood from MDD suicide attempters	Inflammatory/ coagulatory processes
PPP, **MIF, EN-RAGE**, IL1ra, **TNC**, GROa, **vWF,** Prost, LH,	Blood from MDD remitted patients	Cell comunication/ immune system/protein metabolism

AAT, UPA, CathD, HPN, MMP10, FABPA		
Gene Name	**Characteristics of sample**	**Biological process**
CP, CC1QC, **ITIH4**	Blood from MDD remitted patients	Inflammatory process
FGA	Blood from MDD remitted patients	Inflammatory process
CCL11, IFN-γ	Blood from MDD remitted/non remitted patients	Immune system
APOA4, CPB2, C7, CHEK1, ACTN1, **CRP**, THBS1, **FGA**, CFHR5, PYY2, F5, ARFIP1, CFHR2, MYH2	Blood from MDD remitted/non remitted patients	Immune system
vWF,SERPINA1, APOC3, A2M	Blood from responders to CHM	Inflammatory process
VEGFC, Tie2, **BDNF**	Blood from TRD patients	Inflammatory process
SAMP, C4BP	Blood from TRD patients	Complement system
IGF-1, INS, CCL4, **BDNF**	Blood from patients treated with antidepressants and ECT	Several biological processes

Gene Name	Characteristics of sample	Biological process
GRIA1, GRIA2, PRKCγ, PRKCβ, GRIN2B, SLC17A7, GNAQ, CAMK2α, PPP1R1A	Hippocampus from VD animal model	Several processes involving regulation of the nervous system
NSF, ATP5A1, ACO2, STXBP1, DRP-2, SNAP-25	Hippocampus and frontal lobe from early-life stressed animals	Several biological processes
FGF-9, IL-4, **TNF-α** / mTOR, ERK1, PKCα, NSF, SYN1, PACN1, PSD95, NCDN, AATM, COMT, PPP3CC, PKCβ	Serum / hippocampus and frontal cortex from animals treated with ketamine	Several biological processes
AK1, NDK B, HINT1, APT-2, GSTA4 / GSTA6, GSTA4, RAN, Atp5h, Tagln3, Eif5a, SUMO2	Hippocampus from CMS animals / CMS animals treated with oleamide	Energy metabolism, oxidative stress, and cell communication
GFAP, VGLUT1, HOMER1, ATP1A2, UQCRFS1, UQCRC1, PRDX1, PRDX2	Synaptosomes from prefrontal cortex of CMS animals	Cell comunication/ energy metabolism
MMP9, **IL-1**, **CRP**, **TNF-α** / MYPR, MBP, CN37	Serum and frontal cortex of stress-susceptible mice	Inflammatory process/ oligodendrogenesis

3.1 Proteomics findings related to diagnostic biomarkers in drug-naive patients

A relationship among the inflammatory, immune and lipid systems and MDD has been found in some studies in drug-naive patients. These studies contribute to understand the proteome profile without the influence of drug effects. However, the number of studies performed with such groups of patients is small as samples from

drug-naive patients are not easily available. Serum analysis performed by Stelzhammer et al (2014) showed the involvement of the pro-inflammatory proteins ceruloplasmin (CP), RAGE-binding protein (EN-RAGE), ferritin (FTH1), haptoglobin-related protein (HPR), interleukin-1 receptor antagonist (IL-1ra), interleukin-16 (IL-16), macrophage migration inhibitory factor (MIF), serotransferrin (F3) and tenascin-C (TNC), besides two other proteins linked to the oxidative stress process, in first onset patient samples(STELZHAMMER et al., 2014). Another study with drug-naive women identified six differentially expressed proteins, which were apolipoprotein B (APOB), apolipoprotein D (APOD), CP, vitamin D-binding protein (GC), hornerin (HRNR), and profilin 1 (PFN1), that differentiated MDD patients from controls with up to 68% accuracy(LEE et al., 2016). These proteins are related to the immune system, inflammatory and lipid metabolism(LEE et al., 2016). Similar results were found using the combination of heart rate (HR) and plasma proteins in a study conducted by Kim et al (2017) involving proteomics and a machine-learning approach(KIM et al., 2017). Using these techniques, three proteins (in combination with HR) were identified as possible biomarkers for MDD: APOB, CP and GC, which modulates the immune and inflammatory systems(KIM et al., 2017). In another study, using a iTRAQ-based proteomics approach, serum samples from drug-free patients presented significant increases in immune and lipid-related proteins such as C-reactive protein (CRP), inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), serum amyloid A-1 protein (SAA1) and angiopoietin-related protein 3 (ANGPTL3)(WANG et al., 2016). Another study was conducted by Wu et al (2015) using urine samples, in which the enzyme involved in the urea cycle and participating in the nitric oxide metabolic pathway, argininosuccinate synthase 1 (ASS1)(HAINES; PENDLETON; EICHLER, 2011), showed potential as an MDD biomarker(WU et al., 2015).

Efforts have been made to find biomarkers that help psychiatrists differentiate between Bipolar Disorder (BD) and MDD that share changes in the hypothalamic-pituitary-adrenal (HPA) axis(GRUNZE, 2011) and symptoms, such as oscillation of energy levels and mood disturbances, making it difficult to accurately diagnose each disease for proper treatment(PREECE; HAN; BAHN, 2018). Stelzhammer et al (2015) performed the first analysis using LC-MS^E to find differences in protein expression between post-mortem pituitaries of BD, MDD patients and controls. In this study, proteins related to intracellular transport and remodeling of cytoskeletal pathways were found to be altered in MDD patients(STELZHAMMER et

al., 2015b). Furthermore, reduced levels of carboxypeptidase E (CPE) in MDD patient pituitaries suggests that prohormone conversion may be altered, although most hormones did not exhibit altered levels in MDD(STELZHAMMER et al., 2015b). Retinol-binding protein 4 (RBP-4) and transthyretin (TTR) form a complex that is responsible for vitamin A transport and may be involved in mood disorders(FLEMING; NUNES; SOUSA, 2009). Their differential expression have been found from blood samples and may become distinct between BD I (a subtype of BD) and MDD(FRYE et al., 2015). Two other proteins, highly similar to vanin-1 protein (B2RAN2) and endoglin (END), were differentially expressed in plasma MDD drug-naive patients when compared to BD patients(REN et al., 2017).

Suicide attempts are a relevant concern and may affect some MDD patients. One study investigated the plasma of drug-naive depressed attempters (MDD-SA), depressed suicide non-attempters (MDD-NA) and healthy controls using 2-DE-MALDI-TOF/TOF-MS and iTRAQ-LC-MS/M platforms and found alterations in the CRP, SAA1, coagulation factor X (FX), and protein C inhibitor (PCI) in drug-naive MDD-SA group compared with the other groups(YANG et al., 2016). In a validation phase using enzyme-linked immunoadsorbent assay (LISA), six proteins were found differentially expressed relative to both MDD-NA and health controls subjects: tissue factor (TF), coagulation factor II (FVII), coagulation factor V (FV), tissue factor pathway inhibitor (TFPI), activated protein C (APC), and prothrombin fragment 1 + 2 (F1+2)(YANG et al., 2016). Therefore, these results revealed differentially expressed inflammatory and coagulatory proteins. This indicates that further investigations are needed to understand potential mechanisms that may predispose to suicide(YANG et al., 2016).

3.2 Late-life Depression

The term "Late-life depression" often refers to depressive episodes occurring at around 60 years of age or later: the first episode may occur in early years (early-onset depression), or during aging (late-onset depression). It can be described as a heterogeneous and complex neuropsychiatric syndrome, triggered by another serious disease, by comorbidities, or may be the result of drug use(AZIZ; STEFFENS, 2013).

The investigation of the differential expression of peripheral proteins through immunoassays in the blood plasma of elderly patients led to the association between geriatric depression and inflammatory processes and reduction of neurotrophic

support, as well as proteostasis markers and nutrient detection(DINIZ et al., 2016). Some of these same markers suggest that homeostatic dysregulation present in patients with geriatric depression can accelerate the aging process, which can increase the risk of Alzheimer's and dementia(DINIZ et al., 2015). Another important investigation correlated higher SASP index (a set of proteins secreted by different senescent fibroblasts, responsible for inducing the aging of nearby cells) in patients with depression, and may give clues about the molecular mechanisms that lead to LLD(DINIZ et al., 2017). However, there are still few studies which have attempted to investigate the molecular mechanisms and obtain a consistent biological signature of LLD.

3.3 Markers related to response

Biomarkers for antidepressant response represent a strategy to personalize therapy through the characterization of a panel of proteins to guide physicians in the best antidepressant choice for each patient. Serum proteins from patients with current MDD (MDDc) and remitted MDD (MDDr) were compared in one large cohort study from Bot et al (2015). Proteins related to cell communication, signal transduction processes, immune response, and protein metabolism were found to be differentially expressed in serum from MDDc patients compared to that from controls(BOT et al., 2015). Those proteins were pancreatic polypeptide (PPP), MIF, EN-RAGE, IL-1 receptor antagonist (IL1ra) and TNC, growth-regulated alpha protein (GROa) and von Willebrand factor (vWF), a marker involved in homeostasis. When MDDc and MDDr groups were compared, 10 analytes were found to be altered: prostasin (Prost), luteinizing hormone (LH), alpha-1-antitrypsin (AAT), urokinase-type plasminogen activator receptor (UPA), cathepsin D (CathD), hepsin (HPN), matrix metalloproteinase-10 (MMP10), L1ra, vWF, and fatty acid-binding protein adipocyte (FABPA)(BOT et al., 2015).

Regulation of inflammatory processes seems to be associated with a good response to antidepressant treatment. Comparative proteomic analysis of a small cohort of MDD subjects before and after treatment found a decrease of three proteins in serum after remission: CP, complement component 1q (CC1QC; a component of the classic activation) and ITIH4(LEE et al., 2015). Plasma fibrinogen alpha (FGA) levels were the object of investigation between drug responders and nonresponders in another work, without taking into account the type of antidepressant used. High levels

of fibrinogen at baseline were associated with a poor response to antidepressants due possibly to an elevated inflammatory status(MARTINS-DE-SOUZA et al., 2014a).

Investigations of the association between antidepressant treatment response and the immune system have been carried out before and after treatment. In the study of Gadad et al, two proteins demonstrated significant association with treatment response. After 12 weeks of treatment, responders presented higher levels of eotaxin-1 (CCL11), and interferon-gamma (IFN-γ) levels were reduced in nonresponders. Eotaxin may be linked to increased levels of mammalian target of rapamycin (mTOR) protein, related to synaptogenesis. Higher levels of IFN-γ at baseline in nonresponders may be implicated in a higher activation of the kynurenine pathway, which has been associated with MDD, and thus implicating a worse response to medication(GADAD et al., 2017). Similarly, Turck et al conducted a proteome profiling of plasma from MDD patients at baseline and after 6 weeks of treatment. Differences at baseline between responders and non-responders showed significant differences between 29 proteins that could compose a panel of response prediction. Apolipoprotein A-IV (APOA4), carboxypeptidase B2 (CPB2), complement component C7 (C7) and serine/threonine-protein kinase Chk1 (CHEK1) were over 2-fold lower in responders, and alpha-actinin-1 (ACTN1), CRP, thrombospondin-1 (THBS1), FGA, complement factor H-related protein 5 (CFHR5), FV, arfaptin-1 (ARFIP1) and complement factor H-related protein 2 (CFHR2) were over 2-fold higher in responders(TURCK et al., 2017). After 6 weeks of treatment, 18 proteins were observed to have differences of more than 2-fold in responders when compared with the baseline levels. Among these proteins, putative peptide YY-2 (PYY2) and CHEK1 were increased and mysosin-2 (MYH2) was decreased(TURCK et al., 2017).

Integrin and RAS signaling pathways are involved in processes of synaptic signaling and remodeling in the central nervous system and can be potential biomarker candidates for response to antidepressants. Martins-de-Souza et al (2014) compared blood mononuclear cells proteomes at baseline and after 6 weeks of antidepressant treatment, and proteins of integrin signaling pathways were found differentially expressed between responders and nonresponders (MARTINS-DE-SOUZA et al., 2014b). Integrin, RAS and fibrinogens are related to platelet activation with consequent inflammatory response, and N-glycans profiles may distinguish responders from non-responders, since N-glycosylation has a relevant role in platelet activation(PARK et al., 2018). Alterations of coagulation and complement cascades, lipid metabolism, platelet

degranulation and activation pathways were associated to a depressive status, and their changes were associated with response to Chinese herbal medicine (CHM). Proteins such as vWF, epididymis secretory sperm-binding protein (SERPINA1), apolipoprotein C-III (APOC3) and alpha-2-macroglobulin (A2M)were found at different levels in responding patients treated with CHM.(CHEN et al., 2018).

Although many antidepressant drugs are available, there is still a significant proportion of patients who do not respond well to them. Treatment-resistant depression (TRD) is the focus of few proteomic studies. Study from Pisoni et al have demonstrated the role of altered vascular endothelial growth factor-C (VEGFC), angiopoietin-1 receptor (Tie2) and BDNF levels in impaired neurogenesis and neuroplasticity(PISONI et al., 2018). In another study, differentially expressed proteins identified in TRD patients showed altered complement activation, coagulation and lipid transport processes, with more severely affected patients presenting altered serum amyloid P (SAMP) component and the C4b-binding protein (C4BP)(RULAND et al., 2016). Electroconvulsive therapy (ECT) is used to treat TRD patients with higher remission rates than antidepressants drugs, with molecular changes occurring after ECT treatment combined with antidepressants. Although there was an increase of insulin-like growth factor I (IGF-1) and C-peptide (INS), and decrease of MIP-1 beta (CCL4) and BDNF, only levels of C-peptide seem related to symptom improvements(STELZHAMMER et al., 2013). Additional studies are needed to unravel how these molecules are related to the therapeutic role of ECT.

3.4 Animal models

Animal models have been used to investigate the biological mechanisms that lead to depression or even to investigate different responses to drugs. Like other psychiatric disorders, animal models to study depression present limitations in terms of mimicking clinical findings observed in humans. However, animal models have been widely used due to the possibility of investigating biochemical changes in tissues that are not accessible in humans, such as the brain.

The blockade of the bilateral carotid artery has been used to mimic vascular depression (VD). These animals present hippocampal damage and changes in animal weight and behavior similar to depressive symptoms(ZHAO et al., 2018). Hippocampal proteomic analysis showed multiple changes in pathways related to neural plasticity (calcium signaling pathways and neurotransmission), energy and amino acid

metabolism, similar to patients with vascular depression(ZHAO et al., 2018). Nine proteins related to these changes: glutamate receptor 1 (GRIA1), glutamate receptor 1 (GRIA2), protein kinase Cγ (PRKCγ), protein kinase Cβ (PRKCβ), glutamate receptor ionotropic NMDA 2B (GRIN2B), vesicular glutamate transporter 1 (SLC17A7), guanine nucleotide-binding protein G(q) subunit alpha (GNAQ), calcium/calmodulin-dependent protein kinase type II subunit alpha (CAMK2α) and protein phosphatase 1 regulatory subunit 1A (PPP1R1A) were validated by Western blotting and showed reduced expression in the model compared to the sham group(ZHAO et al., 2018).

In a classical model of depression, maternal deprivation, changes were found through synaptosome analysis of hippocampal and whole frontal lobe. This study showed alterations in proteins related to energy metabolism and structural protein disturbances, which suggest that early-life stress may affect cytoskeletal dynamics of synapses. Specifically, vesicle-fusing ATPase (NSF), ATP synthase alpha (ATP5A1), aconitate hydratase (ACO2), syntaxin-binding protein 1 (STXBP1), dystrophin-related protein 2 (DRP-2), and synaptosomal-associated protein 25 (SNAP-25) were found to be differentially expressed(MALLEI et al., 2014).

Studies in rats investigated the proteomic and biochemical alterations of potential antidepressants. Wesseling et al showed that administration of ketamine, an antagonist of the NMDA receptor, promoted changes in the frontal cortex, hippocampus and serum of rodents. Only three serum proteins were affected, fibroblast growth factor 9 (FGF-9), interleukin-4 (IL-4) and tumor necrosis factor alpha (TNF-α), which suggested only minor changes in the peripheral system(WESSELING et al., 2014). Proteins such as the mTOR, extracellular signal-regulated kinase 1 (ERK1), protein kinase Cα (PKCα), vesicle fusing ATPase (NSF), synapsin (SYN1), syndapin-1 (PACN1), postsynaptic density protein 95 (PSD95), neurochondrin (NCDN) were found to be differentially expressed in the hippocampus(WESSELING et al., 2014). Increased levels of mitochondrial aspartate aminotransferase (AATM) occured in the frontal cortex and hippocampus, and lower levels of catechol-O-methyltransferase (COMT) were detected in the hippocampus(WESSELING et al., 2014). In addition, altered levels of calcineurin (PPP3CC) and protein kinase Cβ (PKCβ) were found in the frontal cortex and in the hippocampus after treatment with ketamine(WESSELING et al., 2014). Similarly, rats submitted to chronic mild stress (CMS), a MDD animal model, were treated with oleamide. CMS affected the adenylate kinase isoenzyme protein 1 (AK1), nucleoside diphosphate kinase B (NDK B), histidine

triad nucleotide-binding protein 1 (HINT1), acyl-protein thioesterase 2 (APT-2), and glutathione S transferase A 4 (GSTA4)(GE et al., 2015). Rats subjected to CMS and treated with oleamide presented changes in the levels of glutathione S-transferase A6 (GSTA6), glutathione S-transferase A4 (GSTA4), GTP-binding protein Ran (RAN), ATP synthase subunit d, mitochondrial (Atp5h), transgelin-3 (Tagln3), eukaryotic translation initiation factor 5A-1 (Eif5a), small ubiquitin-related modifier 2 (SUMO2)(GE et al., 2015). Oleamide was associated with increased sucrose intake, showing an antidepressant-like effect, affecting proteins involved in processes such as energy metabolism, oxidative stress and cell communication(GE et al., 2015).

Using the CMS model, a study showed that stress resilient and stress-susceptible rats presented changes in proteins associated with cell interactions and glutamatergic signaling. Glial fibrillary acidic protein (GFAP) and vesicular glutamate transporter 1 (VGLUT1) proteins were found to be decreased in the stress-susceptible group, and proteins linked to ion regulation such as homer protein homolog 1 (HOMER1) and sodium/potassium-transporting ATPase subunit alpha-2 (ATP1A2) were found upregulated in resilient rats(PALMFELDT et al., 2016). Resilient rats have also shown higher levels of antioxidant proteins such as the mitochondrial proteins cytochrome b-c1 complex subunit Rieske (UQCRFS1) and cytochrome b-c1 complex subunit 1 (UQCRC1), related to cytochrome b-c1 complex subunits, and they had lower levels of peroxiredoxins, PRDX1 and PRDX2s(PALMFELDT et al., 2016). Another animal model study investigated the serum and frontal cortex of stress susceptible (SS) and stress resilient mice (SR). This study found that 20 proteins were differentially regulated in the serum and frontal cortex of these groups(STELZHAMMER et al., 2015a). Changes in proteins related to inflammatory system such as matrix metalloproteinase-9 (MMP9), IL-1, CRP and TNF-α were found in serum samples(STELZHAMMER et al., 2015a). Resilient animals presented higher levels of myelin-associated proteins in the prefrontal cortex, including myelin proteolipid protein (MYPR), myelin basic protein (MBP), and 2,3 cyclic nucleotide 3 phosphodiesterase (CN37), suggesting an effect on the oligodendrogenesis process(STELZHAMMER et al., 2015a).

4 Conclusions

Despite many hypotheses that try to explain MDD etiology, there is a lack of defined molecular mechanisms that attempt to relate all main characteristics of this

disorder. Although diagnostic systems such as DSM-V can systemize depressive symptoms, there are overlaps with other psychiatric disorders with respect to both symptoms and biomolecular pathways(GOODWIN, 2015; GOTTSCHALK et al., 2014). Proteomics is a powerful set of techniques that enable the investigation of these biochemical characteristics aiming to propose a panel of diagnosis and response biomarkers.

Proteins such as CP, EN-RAGE, MIF, TNC, IL-1, GC, CRP and SAA1 emerge several times in different studies with the purpose of identifying a molecular profile of MDD. FGA and vWF proteins are recurrent in studies of markers related to treatment response. Changes in the levels of BDNF seem to be related to resistance to treatments. Identification of a trustable and specific panel of markers to MDD can lead to changes for patients, since biomarkers can improve diagnostic capacity and reveal new drug targets, which can be used to develop newer and better drugs. However, there is a missing link between those findings and their validation in clinical practice.

The wide variety of differential expressed proteins may be due the complexity of interactions related to MDD, and there is a need for exploration of possible connections between these pathways. The development of a mathematical model exploring these connections and related symptoms could be useful in this sense. Although the focus of this chapter is proteomics, a field in which much further research is needed, there is also a need for translational and multi-omics research. Given the complexity of MDD, there is a call for proteomics assays that also interact with genomics, transcriptomics, metabolomics, epigenomics, lipidomics and even metagenomics, giving rise to omics-based biomarkers.

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5. CAPÍTULO 2

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Blood plasma high abundant protein depletion unintentionally carries over 100 proteins

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List of abbreviations:

2-D RP/RP	two dimensional reversed phase
DIA	data independent acquisition
HDMS^E	high definition MS^E
HSS	high strength silica
IMS	ion mobility separation
MS^E	tandem mass spectrometry alternating low and high collision energy
UPLC	ultra performance LC

Keywords:

Depletome; High Definition MS^E; Plasma proteome

Abstract

There is a constant interest in blood-based protein biomarkers, which can help to improve diagnosis and treatment outcomes of multifactorial human pathologies. In this regard, proteomic studies usually employ plasma immunoaffinity fractionation to deplete the most abundant plasma proteins, due to the high dynamic concentration range of proteins. The depletion of high abundant proteins allows to obtain less abundant and, oftentimes, more interesting proteins. However, the removal of the fraction of the high abundant plasma proteins - the depletome - may co-elute many unintended proteins due to protein-protein interactions. Little data is available about the depletome and potential protein biomarkers may be lost during this process. To visualize and characterize these proteins, we analyzed the depletome of 20 plasma samples by shotgun mass spectrometry-based proteomics. Thus, using immunoaffinity depletion followed by 2-D LC coupled to an ion mobility-enhanced mass spectrometer, our analysis identified that over 100 proteins are co-eluting with the high abundant fraction. These proteins play roles in several biological processes, such as receptor-mediated endocytosis, complement activation, and regulation of immune response. This study supports that investigating the depletome is important in the quest for biomarkers.

1 Introduction

Proteomics are a set of biochemical tools which can elucidate the role of proteins in the molecular complexity of multifactorial pathologies, including, for example, some types of cancer, psychiatric, neurodegenerative and respiratory diseases (KIM et al., 2016; LEE et al., 2016; MURPHY et al., 2006; TEUNISSEN et al., 2011).These disorders frequently overlap symptoms with other diseases, challenging physicians to find precise diagnostic or adequate treatment. On this matter, biomarkers - a measurable characteristic from an organism’s current state (BIOMARKERS DEFINITIONS WORKING GROUP., 2001; INSTITUTE OF MEDICINE (US) COMMITTEE ON QUALIFICATION OF BIOMARKERS AND SURROGATE ENDPOINTS IN CHRONIC DISEASE, 2014) - are a current focus to improve outcomes and personalize treatments of diseases of multifactorial etiology.

The discovery of novel protein biomarkers can be enhanced by investigation of blood plasma and/or serum, which is a promising human supply of proteins (GEYER

et al., 2016). However, the complexity of blood-based protein samples is a challenge because human blood plasma has a high dynamic concentration range of proteins, stretching over 12 orders of magnitude (ANDERSON; ANDERSON, 2002b; FARRAH et al., 2011). One way to reduce the complexity of blood plasma proteins is by fractionation, discarding the high abundance-fraction after separation by immunoaffinity chromatography. The term “depletome” emerged from the need to investigate the discarded fraction, composed of up to 20 proteins and their isoforms (JAROS et al., 2013a; KOUTROUKIDES et al., 2011). Although depletion technique allows for the identification of many more proteins with low abundance and thusly more potential biomarkers, it can also remove other potential biomarkers due to protein-protein interactions, causing a type of undesired co-immunoprecipitation. Thus, the investigation of depletome requires high sensitivity techniques, such as high-resolution mass spectrometry, that can identify interacting proteins, despite the broad range of concentrations.

Mass spectrometry-based proteomics has potential in dealing with complex protein samples. Furthermore, MS can generate spectra of data from the peptide fragments even with low-abundance proteins (AEBERSOLD; MANN, 2016). An alternative approach called MS^E employs the alternation of collision energy within the fragmentation cell to promote varied molecular fragmentation of ionized peptides (SILVA et al., 2006). Ion mobility separation (IMS) adds another dimension in the separation of the ions, reducing interference and improving the detection capacity of the peaks, making this tool especially suitable for complex samples (DISTLER et al., 2016; HELM; BAGINSKY, 2018) . The association of IMS with MS^E is called $HDMS^E$ and increases the detection of precursor ions from complex samples and confidence in the identification of peptides(DISTLER et al., 2016).

This study aims to increase the knowledge base about the blood plasma depletome, composed by proteins which interact with high abundant fraction. We identified co-eluted proteins of depletome fraction using $HDMS^E$ shotgun mass spectrometry, and after initial identification, we performed *in silico* analysis to characterize which biological processes and molecular functions are involved in that fraction (Fig. 2). Thus, we confirm the relevance of the depletome investigation when the exploration by biomarkers is performed.

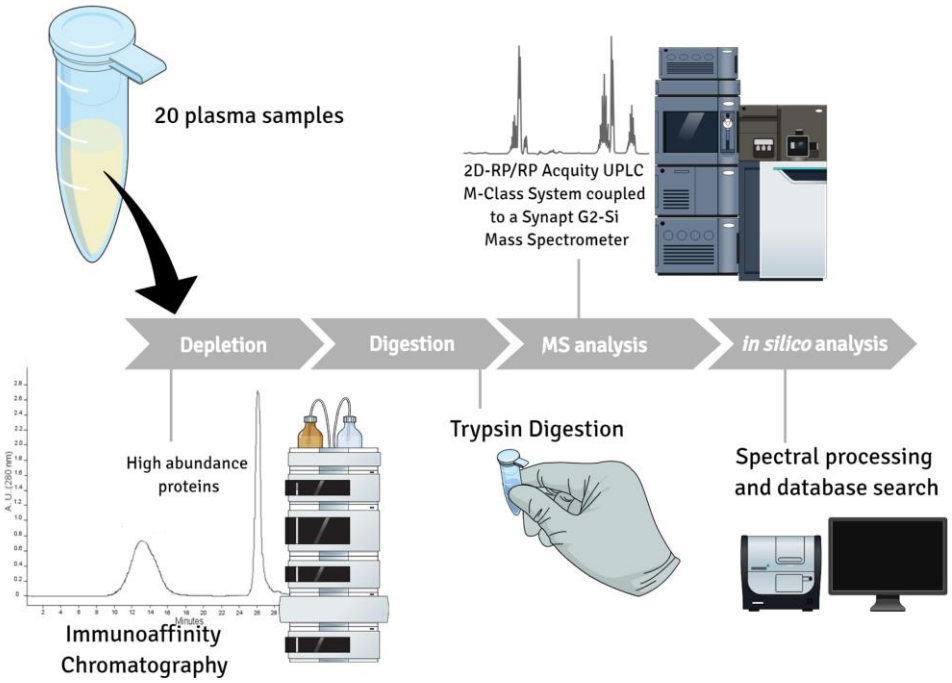

Figure 2: Shotgun workflow. High-abundance proteins bound to the column at depletion step and later eluted. Then, protein samples were digested to peptides with trypsin. The resulting peptides were injected in a UPLC system coupled online to a Synapt G2-Si mass spectrometer with electrospray ionization. Afterwards, proteins were identified and quantified using Progenesis QI against Swiss-Prot Human Proteomic Database.

2 Materials and methods

The cohort for this investigation consisted of 20 human plasma samples. Blood samples were collected at the psychiatric clinic of the University of Magdeburg, Germany as previously described(STEINER et al., 2013), and all participants provided written informed consent. This collection has been approved by the ethics committee of the University of Magdeburg, in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. Shortly after collection, the blood samples were centrifuged for 10 min at 2,000g and the resulting plasma fraction was immediately frozen and stored at -80ºC.

In the depletion step, 30µL of plasma was diluted in 90 µL phosphate buffer (pH 7.4) prepared with 2.5% (v/v) phosphate buffer solution 1M (Sigma-Aldrich), 10.0% (v/v) NaCl 5M and 0.02% (m/v) sodium azide in water. The phosphate buffer was also used to carry the sample into the MARS Hu14 Immunodepletion System column (Agilent Technologies), according the manufacturer's protocol. Then, urea acidic buffer

- 2.0M urea, 0.5M glycine in water, pH 2.25, adjusted with HCl - was used to elute the high abundance proteins bound to the column, releasing the depletome and thus allowing the loading of the next sample. A representative chromatogram of depletion process is represented in Fig. 3A. Depletome buffer was exchanged with 50 mM ammonium bicarbonate using Vivaspin 6 (Sartorius) cartridges. After buffer exchange, proteins were reduced with dithiothreitol (100 mM, 60ºC, 30 min) and alkylated with iodoacetamide (300 mM, 30 min, room temperature, in the dark). Then, we proceeded with protein samples digestion into peptides with trypsin (Promega) at a ratio of 1:100 (w/w trypsin/protein) for 16 h at 37ºC. Digestion was quenched with 5% trifluoroacetic acid for 15 minutes at room temperature. The samples were centrifuged at 20,817g at 6°C for 30 min. The supernatant was recovered and then pH was adjusted using 0.5µL of 1N ammonium hydroxide prior to analysis.

We randomized the samples before mass spectrometry analysis. The peptides were subjected to 2-D UPLC HDMS^E analyses by injection into a 2-D RP/RP Acquity UPLC M-Class System (Waters Corporation) coupled online to a Synapt G2-Si Mass Spectrometer (Waters Corporation). Discontinuous steps of ACN (11%, 14%, 17%, 20% and 50%) were used to perform the first-dimension chromatography using an ACQUITY UPLC M-Class Peptide BEH C18 Trap Column (Waters Corporation). The second-dimension separation column (ACQUITY UPLC M-Class HSS T3, Waters Corporation) was set to acetonitrile gradient from 7 to 85% (v/v) for 36 min at a flow rate of 0.4 µL/min directly into the Synapt G2-Si HDMS. The mass spectrometer was operated in resolution mode with an m/z ratio resolving power of 40,000 FWHM, using ion mobility separation with cross-sectional resolving power of 40 Ω/ΔΩ and data independent acquisition method (DIA). Fragmentation spectra were obtained by MS/MS analysis, performed with a NanoLock Spray (Waters) ionization source in positive ion mode(GARCIA et al., 2017). Representative chromatograms of five fractions related to five steps of acetonitrile and the corresponding spectrum of last fraction is represented in Figures 3B, 3C, 3D and 3E.

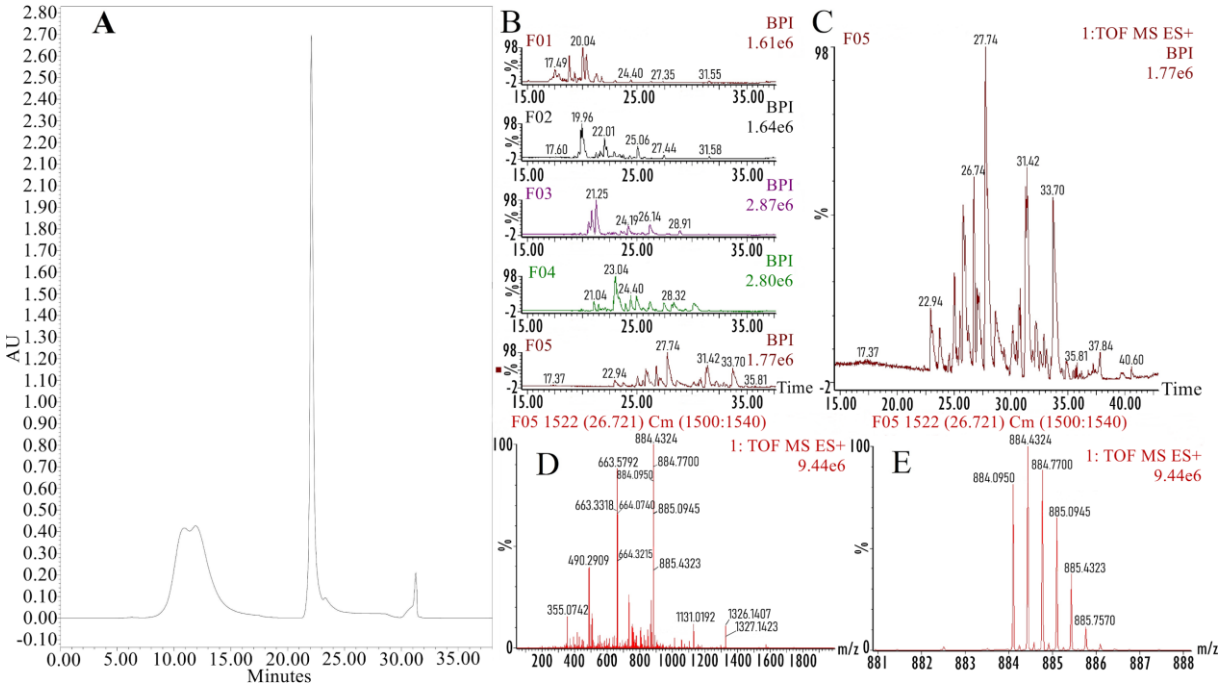

Figure 3: (A) Representative chromatogram of one depletion run. The first peak represents the low abundance proteins and the second peak represents the high abundance proteins or the depletome. (B) Five representative chromatograms of 2-D LC fractionation before MSE analysis. (C) Zoom in on the last chromatogram of figure 3B, related to fraction 5 from that run. (D) Representative spectrum corresponding to data acquisition at the peak at 26.74 of fraction 5. (E) Zoom in on spectrum showed in D.

Afterwards, spectra were processed and proteins were identified and quantified with Progenesis QI for Proteomics® (Nonlinear Dynamics; Waters Corporation; version 4.0) employing Apex3D (Waters) for peak detection and searching against the Swiss-Prot Human Proteomic Database. Quantitation was performed using Hi-N (Hi3). To obtain the preliminary dataset of proteins, the following parameters were considered: trypsin digestion with the maximum of one missed cleavage; variable modification by oxidation (M) and fixed modification by carbamidomethyl (C); false discovery rate (FDR) less than 1%; and mass error less than 20 ppm. In addition, the ion matching requirements were set to select proteins with at least 2 ions per peptide, 5 ions per protein, and 1 peptide per protein. Then, protein grouping was applied, hiding proteins whose peptides are subset of another protein's peptides. Mass spectrometry data were deposited in the ProteomeXchange database and are available under the identifier PXD010273.

Then, the final list of proteins was narrowed down to select proteins identified by at least 2 unique peptides, and proteins whose presence was detected in at least 70% of samples. Keratin and identifications which do not attend these parameters were excluded. Aside from the 20 most abundant proteins and their isoforms(JAROS et al., 2013a; KOUTROUKIDES et al., 2011), bioinformatics analyses were performed on the 81 remaining proteins using DAVID functional annotation tool and the PANTHER Classification System.

3 Results and Discussions

We detected here 12,714 peptides, corresponding to 281 proteins. After protein grouping, 198 proteins remained. Thus, using the Supplementary Table 1 available at “supporting information” of Koutroukides’ paper(KOUTROUKIDES et al., 2011), the comparison between the depletome dataset obtained by Koutroukides and his group and data obtained here revealed 150 non-redundant proteins unique to our study. Although the sum of proteins identified in the two studies were 397 proteins, only 48 are common to both studies (Fig. 4).

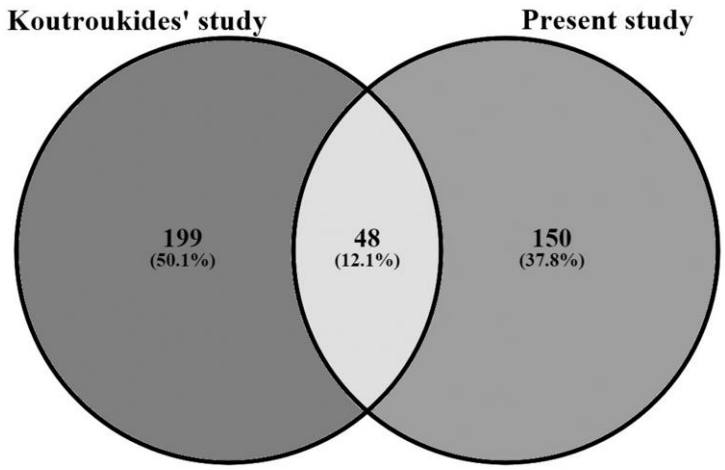

Figure 4: Venn diagram comparing the proteins identified in the Koutroukides’ study and the present characterization.

After stringent filtering, of the 81 low-abundance proteins identified in the depletome, two have experimental evidence of the existence of a transcript, although the existence of protein has not been strictly proven, according to the Swiss-Prot Database: haptoglobin-related protein with Uniprot accession number P00739 and testis- and ovary-specific PAZ domain-containing protein with Uniprot accession

number Q8N9V7. Comparing the concentrations of identified proteins with the highest and lowest abundances, the dynamic range spanned nearly 7 orders of magnitude (Fig. 5).

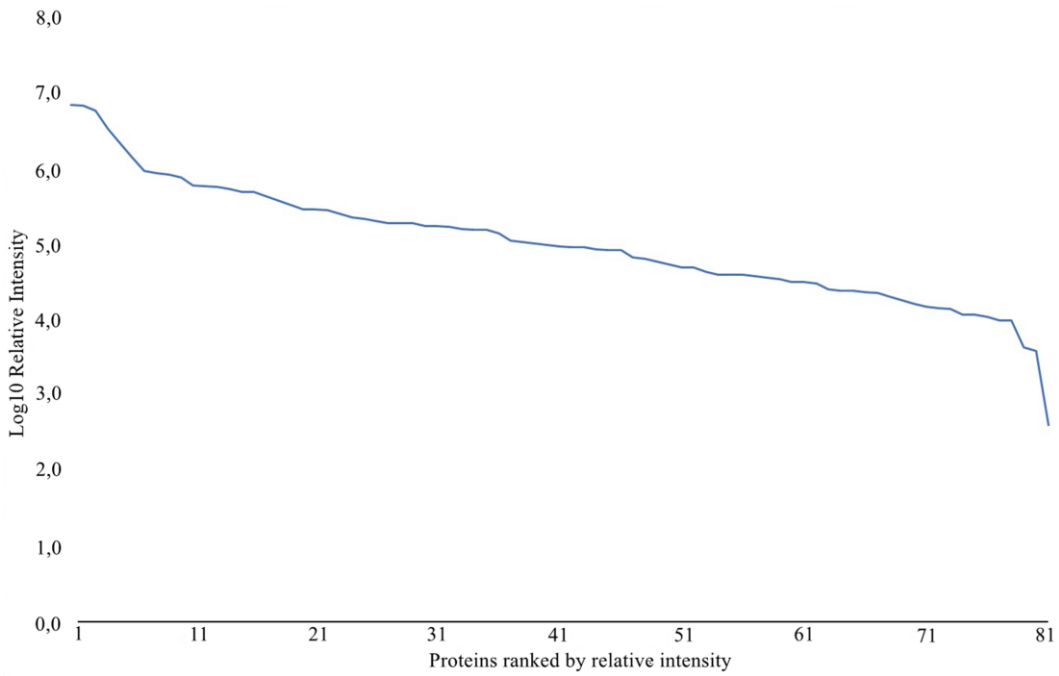

Figure 5: Dynamic range of quantified proteins.

Results from the DAVID functional annotation chart using the GOTERM Direct category (p-value<0.01) reported 35 main biological processes (Fig. 5). Receptor-mediated endocytosis was the biological process with higher number of associated proteins - 19 proteins of 81 low abundance proteins (23%) were associated with this biological process. Fourteen different molecular functions (Fig. 6) were associated with low abundance proteins found in the depletome. Some identified proteins were not able to be classified in terms of biological processes (7%) and molecular functions (11%), according to the PANTHER Classification System.

Thus, this study complements the dataset obtained by Koutroukides et al. (2011), by using a similar methodological approach. There is a minor overlap of identified proteins between both studies, which may be related to the differences of depletome acquirement. Although the two studies present limited comparability of results, the different identifications between both studies may also be associated to depletome complexity.

Proteins which participate in receptor-mediated endocytosis, and negative regulation of endopeptidase activity biological processes were found in the depletome

(Fig. 7). Alterations in endocytic mechanisms controlling traffic of lipids and proteins may be involved in the onset of several diseases, such as psychiatric and immune-related disorders, and some types of cancer (IMAMURA, 2019; SCHUBERT et al., 2012; WILSON; VILLADANGOS, 2005). Moreover, endocytic mechanisms can be explored with the purpose of personalized delivery of drugs, improving outcomes of leukemic and mesenchymal cancers and other disorders (GONDA et al., 2019; NIELSEN et al., 2017; SILVA et al., 2018). However, endocytosis pathways regulation remain to be fully understood even after 40 years since its discovery (GOLDSTEIN; ANDERSON; BROWN, 1979; SCHMID, 2019). Therefore, more in-depth investigation into the role of the depletome proteins in several disturbances can bring important insights into the biochemical pathways related to diseases and patient response to medication.

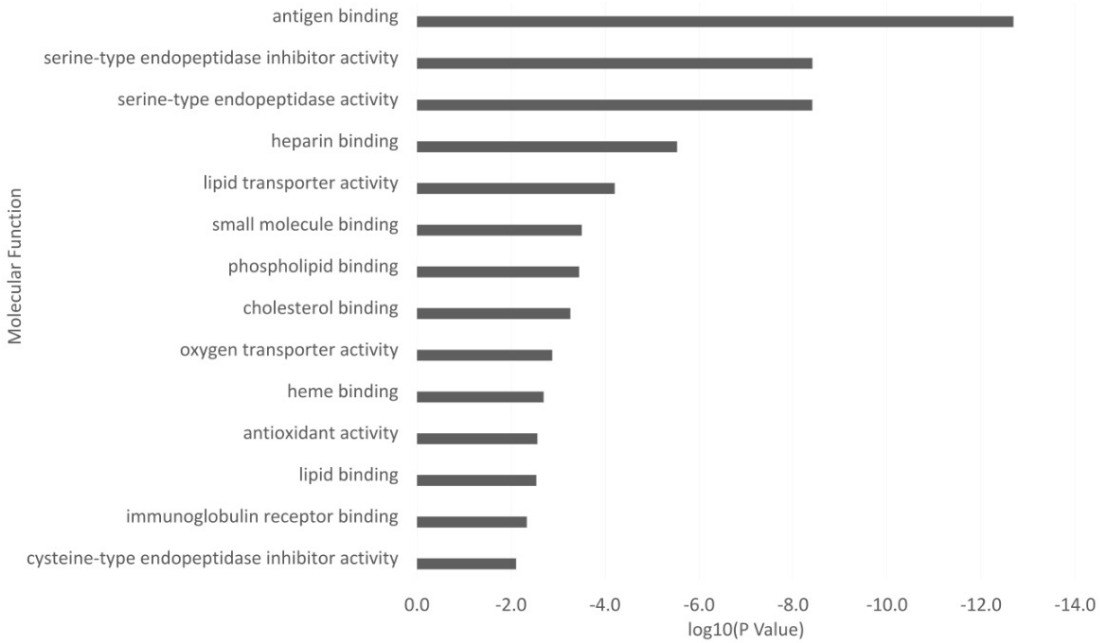

Figure 6: Enrichment analysis expressed in p-value from the DAVID functional annotation chart using GOTERM Direct category reported 14 different molecular functions.

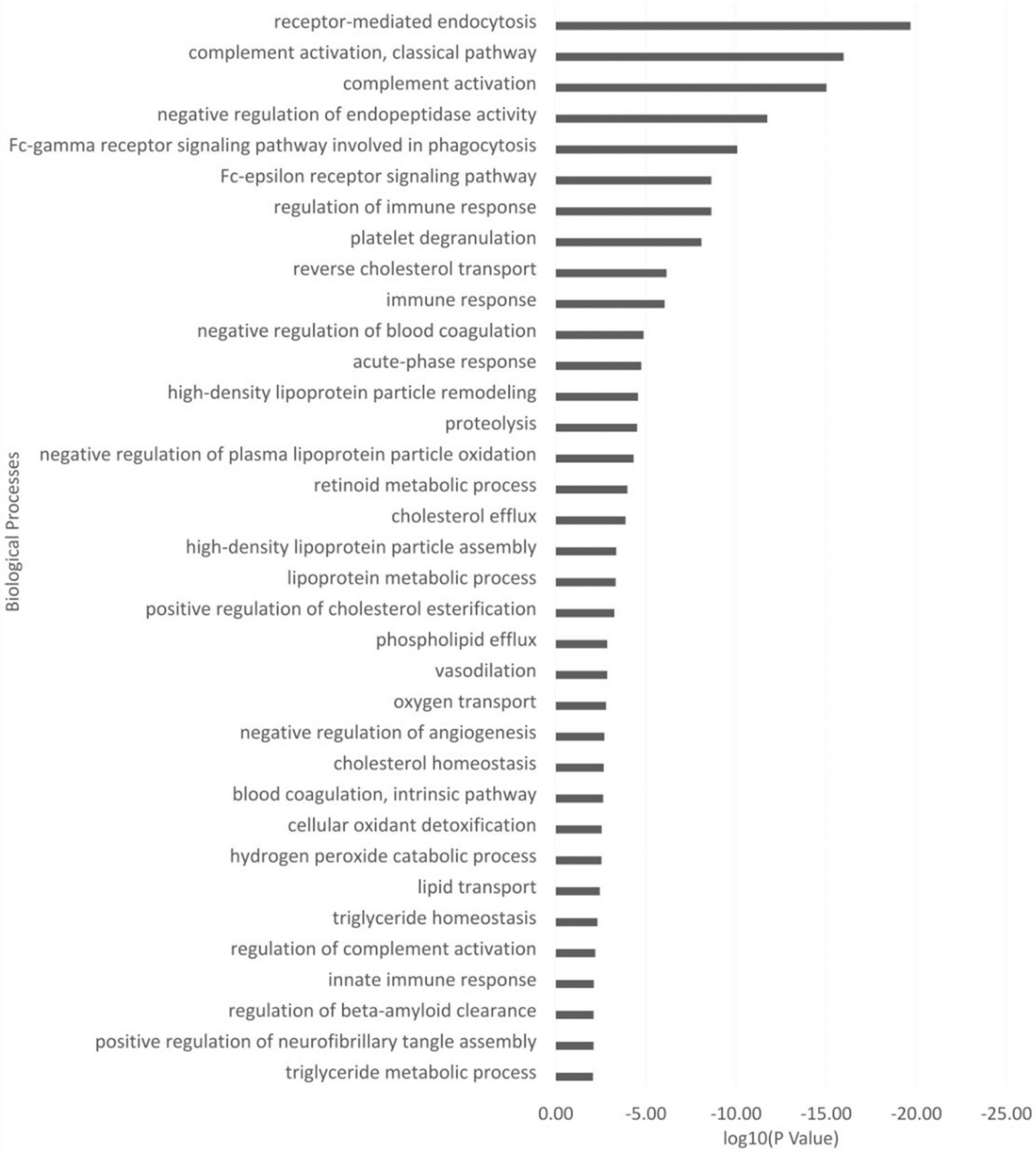

Figure 7: Enrichment analysis expressed in p-value from the DAVID functional annotation chart using GOTERM Direct category reported 35 main biological processes.

Another important feature of the depletome is that it includes proteins related to immune processes and the complement system. Thus, investigation of the depletome can bring insights about autoimmune, metabolic, neurodegenerative and psychiatric diseases (DE LUCA et al., 2018; GIBNEY; DREXHAGE, 2013; MADDA et al., 2018; MORENO-NAVARRETE; FERNÁNDEZ-REAL, 2019; TOMASIK et al., 2016). Several studies suggest that certain changes in the central nervous system may be caused by an imbalance of the peripheral immune system (ZENI-GRAIFF et al., 2016), possibly due to a blood-brain barrier rupture and consequent disturbance of the hypothalamic-pituitary-adrenal axis(WATANABE; SOMEYA; NAWA, 2010). Approximately 20 proteins involved with immune response and activation of the complement system were found in the depletome, making this fraction a source of possible biomarkers related to the neurodegenerative and psychiatric disorders.

4 Concluding Remarks

In future studies, it would be necessary to obtain a better resolution of the depletome fraction, since very large quantities of some proteins such as albumin interfere with the detection of the proteins with which they interact. One potential solution to the problem of low-abundance proteins co-eluting with the depletome could be to create different pH steps or a gradient instead of releasing the depletome all at once. Standardization with mass spectrometry analyses would determine if some co-eluting proteins can dissociate from the column at intermediary pH values without releasing the most abundant proteins from the column.

Considering the intrinsic potential of plasma to participate in and exhibit changes in response to endogenous and exogenous stimuli, plasma fractions are excellent samples to search for biomarkers related to several disorders. Outlook regarding diagnosis-dependent differential expression of proteins in the depletome could be interesting for future research, when looking for potential diagnostic or therapeutic potential biomarkers.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data Availability Statement

The mass spectrometry proteomic datasets for this study can be found in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD010273.

Author Credit Statement

Licia C. Silva-Costa: Formal Analysis, Investigation, Writing – Original Draft. Sheila Garcia-Rosa: Methodology, Investigation. Bradley J. Smith: Writing – Review & Editing. Paulo A. Baldasso: Methodology. Johann Steiner: Resources, Writing – Review & Editing. Daniel Martins-de-Souza: Conceptualization, Supervision, Writing – Review & Editing.

6. CAPÍTULO 3

Manuscrito em preparação a ser publicado como parte integrante do livro *Shotgun Proteomics: Methods and Protocols* que faz parte da série de livros *Methods in Molecular Biology*, publicados pela Springer Nature.

HUMAN BLOOD PLASMA INVESTIGATION EMPLOYING 2D UPLC-UDMS^{E} DATA-INDEPENDENT ACQUISITION PROTEOMICS

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Abstract:

Proteomic tools are especially useful when it comes to investigating complex samples such as human blood plasma, in which protein quantities can span across up to ten orders of magnitude. Ultra Definition Mass Spectrometry, in combination with two-dimensional liquid chromatography, provides better coverage of complex proteomes, and allows for better control of collision energy, keeping the

fragmentation benefits of high collision energy associated with drift time measurements from ion mobility separation. Here, we present a protocol to assist in the identification of proteins in human blood plasma and other similar samples with a large dynamic range.

Key words: multifactorial disorders, immunoaffinity depletion, tryptic digestion, serum, depletion, mass spectrometry

1 Introduction

Several human diseases are considered ”multifactorial”, given the complex interaction of genes, their direct (RNA), and indirect products (proteins, including their modifications and metabolites) and the environment. Such complexity makes multifactorial diseases challenging for researchers and physicians. Understanding how biological mechanisms and changes in gene expression are implicated in these diseases is a long journey since it is imperative to bring together data and analyses from multiple areas and fields of study such as genetics, epigenetics, analytical chemistry, statistics, mathematical models, among many others. Due to the complex nature of multifactorial diseases, the use of personalized medicine is becoming more important in the research and medical fields. Overall, goals of personalized medicine include the development of reliable prediction models of individual risk as well as knowledge about therapeutic outcomes (CAMPBELL; LI; SHAM, 2018; GUEST; GUEST; MARTINS-DE-SOUZA, 2016). Other goals include establishing diagnostic and prognostic molecular signatures or biomarkers for different diseases and stratifying individuals by symptoms or response to treatment (GUEST; MARTINS-DE-SOUZA, 2017; PATEL, 2014).

High-throughput shotgun proteomics, a powerful tool for the investigation of multifactorial diseases, can be employed to study these topics as it can identify and quantify the proteins that are present in samples at a certain point of time (AEBERSOLD; MANN, 2016; WU; MACCOSS, 2002) . Such broad acquisitions are able to reveal possible alterations that cause -- or are a result of -- diseases or treatments. Furthermore, molecular signatures or biomarkers can be proposed and confirmed by proteomic investigations using non-invasive samples such as blood, along with other tissues (AEBERSOLD; MANN, 2016; JAIN, 2016).

Human blood is relatively easy to obtain. Proteins present in human blood plasma or serum range over ten orders of magnitude (ANDERSON; ANDERSON, 2002a; PIEPER et al., 2003; SCHWENK et al., 2017; TIRUMALAI et al., 2003). Furthermore, these proteins may have the potential for diagnosis due to the direct contact between blood and all internal organs (ANDERSON; ANDERSON, 2002a; SCHWENK et al., 2017). Complicating analyses, however, is the fact that 99% of the total plasma protein mass consists of well-known plasma or serum components -- considered the high-abundance fraction -- which can impair the visualization of the low-abundance protein fraction (GIANAZZA et al., 2016; TIRUMALAI et al., 2003). The low-abundance fraction is composed of many proteins with relatively low concentrations and is an important source of potential biomarkers. Thus, the depletion of the major proteins of the plasma/serum (as shown in Fig. 8) improves the detection, identification, and quantitation of low-abundance proteins (ROCHE et al., 2009; SMITH et al., 2011). Several techniques for the depletion of human blood plasma exist but immunoaffinity chromatography fractionation prior to mass spectrometry analysis represents the state-of-the-art in this matter, serving to increase the analytical power of sample analysis (GARCIA et al., 2017; JAROS et al., 2013c; SILVA-COSTA et al., 2019; TU et al., 2010).

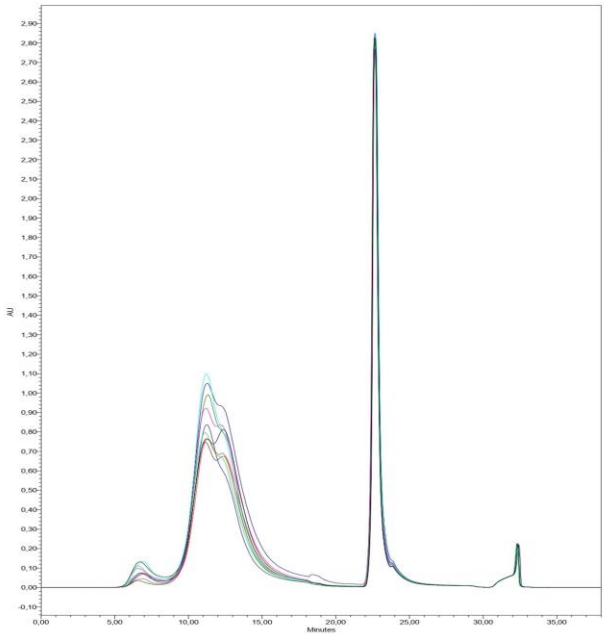

Figure 8: Representative chromatogram of 10 samples depleted by immunoaffinity chromatography. The first peak corresponds to the low abundance fraction; the second peak corresponds to the high abundance fraction, and the third peak corresponds to the buffer change.

When high-performance liquid chromatography (LC) coupled online with mass spectrometry emerged, it gave rise to shotgun proteomics, a type of analysis where a mixture of digested proteins -- peptides -- are separated by reverse-phase chromatography (LINK et al., 1999a) before their mass is analyzed. Mobile phase gradients allow molecules present in complex mixtures to be eluted gradually, improving chromatographic resolution. This fractionation allows the wide range of peptides present in complex mixtures -- including low-abundant, blood plasma, protein digests -- to be separated due to their different retention times by the stationary phase in the column. Then, in classic proteomic study design, the peptides are ionized and conveyed to fragmentation in the mass spectrometer (LINK et al., 1999b).

The fragmentation is used to acquire information on the mass-to-charge ratio of ionized precursor ions and the product of fragmentation ions. MS^E mode alternates low and high collision energy to provide, simultaneously, information about precursor and product ions. An additional dimension of separation for co-eluted peptides can be obtained by using ion mobility separation, a technique named $HDMS^E$ acquisition (GEROMANOS et al., 2009, 2012; PRINGLE et al., 2007). Ion mobility spectrometry is based on the separation of ions according their shape and size, using their drift time (dt), as they cross the gas chamber at different rates. Thus, as the ions move through a gas ion optics chamber under positive pressure and an electric field, their mobility will not only depend on their mass-to-charge ratio but also the conformation of the ions, as molecules with a larger surface area will interact and lose kinetic energy, then moving slowly through the gas chamber (HELM et al., 2014; LANUCARA et al., 2014).

Although the $HDMS^E$ method present advantages relative to the amount of identified peptides, some precursor ions may remain unfragmented, since the collision energy increases linearly during the high collision energy phase. Thus, the adjustment of collision energy parameters to an optimum non-linear gradient of energy to a reference sample over the fragmentation cycle was established as a method called Ultra Definition Mass Spectrometry ($UDMS^E$). $UDMS^E$ provides better coverage of complex proteomes and allows for better control of collision energy, while still keeping the fragmentation benefits of a traveling-wave ion mobility mass spectrometry (DISTLER et al., 2014, 2016; SOUZA; GUEST; MARTINS-DE-SOUZA, 2017). $UDMS^E$ has been shown to improve the efficiency of fragmentation, increasing peptide

identification rates, and consequently leading to greater sample coverage (DISTLER et al., 2014). Also important, as in several other methods, UDMS^E is compatible with relative quantitation, since the relationship between MS signal response and protein concentration can be used to compare protein abundance across analyzed samples, a fact that allows the identification of potential protein biomarkers (SILVA et al., 2006).

UDMS^E can contribute to improve the body knowledge of multifactorial diseases and propose potential markers, by obtaining a reliable identification of a high number of proteins across different groups of human blood plasma. There are still few studies in both individual and pooled samples employing UDMS^E to investigate biological and molecular mechanisms of complex diseases or potential biomarkers of diagnosis or response to treatment. Thus, we present a step-by-step protocol to assist in the identification of proteins in human blood plasma, to contribute to possible further studies using such technology.

2 Materials

2.1 Liquid Chromatography Equipment and Software

1. HPLC system (Waters Corporation),
2. Absorbance Detector (Waters Corporation),
3. Injection system,
4. Buffer solution A: 40 mM sodium phosphate (pH 7.4), 0.5 M NaCl, 0.02%, sodium azide,
5. Buffer solution B: 2 M Urea, 0.5 M glycine (pH 2.25),
6. Empower Pro 2 software (Waters Corporation),
7. MARS Hu14 column (Agilent technologies) (4.6 mm inner diameter, length 100 mm, capacity of 40 µL of plasma or serum).

2.2 Mass Spectrometry Equipment and Software

8. Waters Total Recovery vials with preslit PTFE/silicone caps,
9. Acquity UPLC M-Class with 2D RPxRP technology (Waters Corporation),
10. Synapt G2-Si Mass Spectrometer (Waters Corporation),
11. Acquity UPLC M-Class Peptide BEH C18 Trap Column (130 Å, 5 µm, 300 µm× 50 mm) (Waters Corporation),

12. Acquity UPLC M-Class Symmetry C18 Trap Column, 2D (100 Å, 5 µm,
180 µm × 20 mm) (Waters Corporation),
13. Acquity UPLC M-Class HSS T3 Column (1.8 µm, 75 µm × 150 mm) (Waters
Corporation),
14. NanoLock Spray dual electrospray ion source (Waters Corporation),
15. 100 fmol/uL human [Glu^1]-fibrinopeptide B human (Glu-Fib),
16. Solvent C: 20 mM ammonium formiate (pH 10),
17. Solvent D: 100% acetonitrile (ACN),
18. Solution E: 0.1% formic acid in water,
19. Solvent F: 0.1% formic acid in ACN,
20. UniProt human proteomic database,
21. Masslynx Mass Spectrometry Software®,
22. Progenesis® QI for Proteomics version 4.0+ (Waters Corporation),
23. Cytoscape software,
24. Ingenuity Pathways Analysis software.

2.3 Sample Preparation and Digestion

25. Human serum or plasma samples, or other similar samples,
26. 0.5 and 1.5 mL Eppendorf LoBind tubes,
27. 50 mM ammonium bicarbonate solution (pH 7.5),
28. Centrifugal Filter Units for 4 mL sample with 3000 Da molecular weight cutoff (MWCO)(Amicon® Ultra-4 Centrifugal Filter Units),
29. Refrigerated bench-top centrifuge with rotor for 15 mL conical tubes,
30. Spectrophotometer with UV lamp *(see Note 1).*
31. 0.2% solution of RapiGest™ SF Surfactant (Waters Corporation),
32. 100 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate solution (pH 7.5),
33. 300 mM iodoacetamide (IAA) in 50 mM ammonium bicarbonate solution (pH 7.5),

34. Sequencing grade modified trypsin (Promega Corporation),

35. Trypsin resuspension dilution buffer (Promega Corporation),

36. Heater or thermoshaker,

37. 5% trifluoroaceticacid (TFA),

38. MassPREP™ Enolase digestion standards (ENO) (Waters Corporation).

3 Methods

3.1 Human Plasma or Serum Protein Depletion

1. Prepare the buffer solutions A and B for the HPLC system (*see Note 2)*.
2. Dilute 40 µL of human plasma sample using 120 µL Buffer A and centrifuge the sample for 15 min at 21,000 x g.
3. Transfer the supernatants to microtubes and keep them on ice.
4. Connect buffer A and buffer B flasks to pump inlets and purge the HPLC system at 1 mL/min for 5 min (without a column).
5. Set the depletion method provided for column's manufacturer using Empower Pro 2 Software *(see Note 3)*.
6. Run the HPLC method once without a column.
7. Attach the Agilent MARS Hu14 column to the HPLC system.
8. Equilibrate the column with buffer A for 30 min *(see Note 4)*.
9. Inject 120 µL of sample into the HPLC loop and start the LC method *(see Note 5)*.
10. Run the chromatographic method for depletion and collect eluted fractions using 1.5 mL tubes.
11. Store collected fractions at -80°C until further analyses.

3.2 Desalting and Buffer Exchange

1. Wash sample concentration tubes with ultrapure water.
2. Add 3 mL of 50 mM ammonium bicarbonate and centrifuge at 6,600 x g for 15 min.

3. Add the low abundance fraction samples to each concentration tube *(see Note 6).*

4. Centrifuge at 6,600 x g for 17 min or until the remaining volume in the upper chamber is around 300-600 µL.

5. Remove the assembly and discard the flow through.

6. Complete the sample volume to 4 mL using 50 mM ammonium bicarbonate solution.

7. Concentrate the sample by centrifuging at 6,600 x g for 30 min or until the remaining volume is around 300-600 µL *(see Note 7)*.

8. Mix the concentrated sample with an up-and-down pipette motion several times and transfer it to a 1.5mL tube *(see Note 8)*.

3.3 Tryptic Digestion Using RapiGest^{TM} SF Surfactant

1. Preheat a block heater or thermoshaker to 80°C.
2. Add 50 µg of sample (1 µg/µL) to 1.5 mL microtubes *(see Note 7)*.
3. Add 10 µL of 50 mM ammonium bicarbonate (pH 8.5) and mix well by inverting.
4. Add 25 µL of 0.2% *Rapi*Gest SF and mix well.
5. Heat microtubes using a block heater or thermoshaker at 80°C for 15 min.
6. Remove the tubes from the heater or thermoshaker and centrifuge briefly to remove any condensation on the tube walls and lid. Set the temperature to 60°C.
7. Add 2.5 µL of dithiothreitol solution and mix.
8. Heat microtubes using a block heater or thermoshaker at 60°C for 30 min.
9. Remove the samples from the block heater or thermoshaker and allow them to cool to room temperature.
10. Add 2.5 µL of iodoacetamide solution and vortex to mix.
11. Incubate the samples for 30 min at room temperature in the dark.
12. Add 10 µL of trypsin solution and vortex *(see Note 9).*
13. Incubate the samples at 37°C overnight *(see Note 10)*.
14. Add 10 µL of 5% TFA to hydrolyze the RapiGest and mix by inversion.

15. Incubate the samples at 37ºC for 90 min.

16. Centrifuge the samples at 20,800 x g and 6°C for 30 min.

17. Transfer the supernatants to Waters Total Recovery vials.

18. Add a proportional amount of enolase so that each injected fraction has 0.5 µg of protein digest and 80 fmol/µL of enolase.

3.4 HDMS^E Spectra Acquisition and Scouting Runs

1. Establish solutions C and D as mobile phases of first-dimension chromatography.

2. Set up solutions E and F as mobile phases of second dimension chromatography.

3. Calibrate the mass spectrometer according to the manufacturer's instructions.

4. Set the MS acquisition method to alternate between low and high energy, with no selection window and a continuous ion current.

5. Use a CE ramp from 19 eV to 53 eV in the transfer cell for the elevated energy MS scan.

6. Set Nanoflow conditions on the Tune Page.

7. Set the mass spectrometer to operate in resolution mode with an m/z ratio resolving power of at least 40,000 FWHM, using ion mobility with a cross-section resolving power of 40 Ω /ΔΩ.

8. Adjust the first dimension chromatography method on an ACQUITY UPLC M-Class Peptide BEH C18 Trap Column to perform the fractionation through five discontinuous steps of acetonitrile (11.4%, 14.7%, 17.4%, 20.7% and 50.0%) of 10 min each at a flow rate of 2 µL/min.

9. Adjust the second dimension separation using an ACQUITY UPLC M-Class HSS T3 Column using an acetonitrile gradient from 7% to 40% (v/v) over 54 min at a flow rate of 0.4 µL/min directly into a Synapt G2-S*i* HDMS.

10. Load 1uL of 1ug/uL samples into an ACQUITY UPLC M-Class 2D System coupled to Synapt G2-S*i* HDMS and perform HDMS^E acquisitions *(see Note 11)*.

11. Access each chromatogram and, using the "Integrate Peaks" tool, determine the relative proportion of sample volume for the next steps (*see Note 12*).

3.5 Selection Rules and UDMS^E Acquisition

1. Load Drift Scope software.
2. Using a selection tool, trace a line over the $[M + 2H]^{2+}$ charge region (line A of Figure 9) and then, trace a line under the $[M + 2H]^{2+}$ charged region (line B of Figure 9) (*see Note 13*).
3. Export the selection rule as .rul file without cropping the data (*see Note 14*).
4. In the MassLynx window, edit the existing HDMS^E method correspondent to the first fraction *(see Note 15)*.
5. Double click on the yellow bar.
6. Open the mobility tab, check the “Apply Rule file for Change State/Drift Time Shipping” box and open the selection rule file previously saved.
7. Repeat those steps for the other fractions for each sample. This step is time-consuming but ensures the best quality data.
8. After establishing the method for each fraction of each sample, re-run the samples using their new, *quasi-specific* collision energies drift times-method and loading their relative volume previously calculated during step 3.4.11.

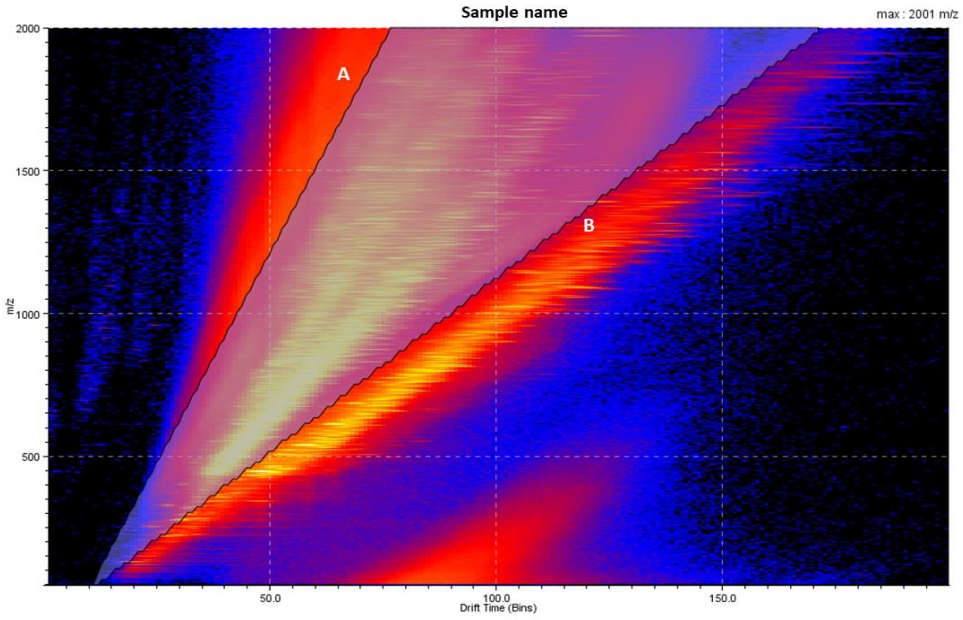

Figure 9: Selection of the multiple charged state region (A) excluding the region above the $[M + 2H]^{2+}$ charged state; (B) Excluding the region under the $[M + 2H]^{2+}$ charged state.

3.6 Data Processing, Quantification and Identification

1. Load Progenesis® QI for Proteomics (version 4.0+) software to process LC-MS/MS information (raw data).
2. Select and import the raw data for the first fraction, provide a lock mass of *m/z* 785.84261 to perform the calibration. Select an appropriate *.fasta databank for threshold calculation.
3. After all files of the same fraction have been imported, click on “Start Automatic Processing” and select to assess all runs in the experiment for alignment suitability.
4. Check the box “Yes, automatically align my runs” and click ”Next”.
5. In “Perform Peak Picking” click in “Set Parameters Up”. At “Runs For Peak Picking” tab, ensure all runs are selected
6. Ensure the sensitivity is set to default, and keep peak picking on automatic at the “Peaking Limits” tab. Keep the chromatographic peak width box unchecked.
7. Set the “Maximum Charge” to 5.
8. Click “OK” to close the “Peak Picking Parameters”.
9. In the “Set Up An Experiment Design” window, click “Next”.
10. On the “Identify Peptides” tab, verify if the “Use MS^E data from my runs to identify peptides” is selected and click to set parameters.
11. Under “Enter the search parameters”, select an appropriate database.
12. For “Common search parameters”, select trypsin as “digest reagents”, 1 maximum as “missed cleavages”, 600 kDa as “max protein mass”, carbamidomethyl C as a fixed modification and methionine oxidation as a variable modification.
13. For “Search tolerance parameters”, select FDR less than 1% (*see note 16*).
14. For “Ion matching requirements”, select 2 fragments per peptide, 5 fragments per protein, and 1 peptide per protein. Click to save parameters and then click next.

15. Under the “Protein Quantitation” tab, select Relative Quantitation Using Hi-N option and choose “3” as the number of peptides to measure per protein. Check the box “Use protein grouping” (*see Note 17*).

16. Click “Finish” to start automatic processing *(see Note 18)*.

17. Under the “Experiment Design Setup” tab, the samples must be grouped into one condition, corresponding to each analysed fraction *(see Note 19)*.

18. Under the“Refine Identifications” tab, filter for and remove peptides which have an absolute mass error greater than 20 ppm.

19. After processing each of the 5 fractions, load Progenesis® QI for Proteomics software, and under the tab “Combine Analysed Fractions”, click “Recombine analysed fractions”.

20. Select the five fractions to recombine, import the selected experiments, and put them in ascending order. Click on “Section Complete”.

21. Rename each sample if needed, select whether the experiment design is between-subject or within-subject design and group samples according to their experimental groups.

3.9 In Silico Analysis, Available Tools

1. Cytoscape, Reactome, DAVID and Panther are useful and free applications and tools that help analyze and visualize pathway enrichment, gene ontology, and molecular investigation.

2. Ingenuity Pathway Analysis, which is paid software, is able to perform pathway and functional correlation analyses.

4 Notes

1. We employed the A280 method of quantification using a DS-11 Spectrophotometer (Denovix Corporation), but the BSA method or fluorometric methods can be used.

2. Information about column variance may be required. Depletion or addition of known amounts of a non-human protein standard may allow to evaluate the efficiency of the column.

3. Empower Pro 2 Software is used to process data and recording.

4. Wash the sample loop with buffer A with at least 3 times the sample volume between the runs.

5. Take care to avoid air entering into the system through the syringe, if manual injection is employed.

6. Take care to observe and adjust the volume to equalize volumes in the concentrator tubes, if it is needed.

7. A method to quantify proteins is required to proceed with this protocol. After the desalted sample is concentrated, before removing the sample from the concentrator, its concentration can be monitored to surpass 1 µg/µL, allowing for dilution to 1 µg/µL with ammonium bicarbonate (50 mM). Avoid heating the sample over 4 ºC to prevent protein degradation.

8. To prevent ionic suppression, steps 4-7 can be repeated.

9. This is a 1:100 wt:wt ratio of enzyme:protein.

10. Incubate the samples for at least 12 h and up to 18 h maximum.

11. Distribute pooled or individual samples or quality controls (QCs) according to your experimental design. We prefer to use individual and randomized samples alternating with QC injections.

12. When opening the chromatogram, open a time point near the beginning of the elution and move forward in time until background noise is washed out by peptide signal. Do the same for the end of the elution period, moving backwards. These should be used as the start and end points for the integration range. The total area under the curve can then be compared between samples to create ratios of peptide signal to calculate any fine-tuning adjustments necessary due to undigested proteins and other contaminants.

13. Remember to maximize the software window, and be careful to note cross the lines under multiple charged state and over multiple charged state.

14. It is recommended that the file be checked using an appropriate text editor to verify that the file was saved properly. On rare occasions, rule files are saved empty.

15. Save as UDMS^{E} method before changing. This ensures that the original method is not overwritten.

16. An FDR of 1% can be selected here.

17. Ungrouping proteins may provide more information, however the data is more complex and needs more processing to get the desired answers. Deselect this if the collaborating bioinformatician, or biostatistician requires.

18. The processing must be done for each of the fraction groups before combining the fractions (using a built-in tool with Progenesis) and proceeding with the experiment.

19. This procedure must be done for each of the 5 fractions.

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7. CAPÍTULO 4

Manuscrito em preparação.

Employing systems biology-based proteomics to understand late-life depression

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Abstract

Depression is a complex and multifactorial disease and is a comorbidity in severe pathologies that are common in the elderly. The peculiarities of depression in adults over 60 form a heterogeneous syndrome called late-life depression (LLD), which affects about 6.5% of the world's elderly population. LLD can contribute to a deterioration in general health, leading to a significant risk of cognitive impairment, vascular dementia, and Alzheimer's disease. Despite all previous studies related to aging and its disorders, there is still limited information about the molecular mechanisms of LLD and how they are related with those disorders. Therefore, we

analyzed the blood plasma of 50 older adults through untargeted mass spectrometry and used systems biology tools to identify biochemical pathways and biological processes affected by the disease. We found 96 differentially expressed proteins between LLD patients and control individuals that confirm an association between LLD and an overall upregulation of complement cascade; but we also found downregulated pathways of the innate immune system, such as classical antibody-based complement activation and activation of C3 and C5. We also compared the protein expression between patients to delineate the proteomic profile related to cognitive impairment and severity of depression. These data can help to build understanding of the molecular basis of LLD, allowing an integrated view of biomolecular alterations that occur in this disorder.

1. Introduction

Depression (Major Depressive Disorder or MDD) affects 4.4% of the world's population and is one of the leading causes of suicide("WHO | Depression and Other Common Mental Disorders", 2017). According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM 5), this disorder is characterized by depressed mood or anhedonia, significant weight loss or gain, sleep changes, psychomotor alterations, fatigue, guilt, impaired ability of concentration and suicidal ideations. The diagnosis of depression occurs when five or more of these symptoms are present for at least two weeks, and at least one of the symptoms must be depressed mood or loss of interest("American Psychiatric Association Diagnostic and Statistical Manual of Mental Disorders (DSM-IV)", [s.d.]).

Depression is one of the major psychiatric comorbidities that arise during other serious disorders, including age-related illnesses, and it is common to observe a coexistence with Alzheimer's and Parkinson's disease, along with an overlap of symptoms resulting from cognitive decline in this disease (LYKETSOS; OLIN, 2002a; SANTIAGO; BOTTERO; POTASHKIN, 2017; WEINTRAUB et al., 2007). Depression symptoms may also be associated with regulatory patterns of gene expression that lead to a higher occurrence of Alzheimer's(LUTZ et al., 2020). Along these lines, cognitive impairment is a common condition in adults with depression, and depressive symptoms are common in elderly people diagnosed with Alzheimer's. Both Alzheimer's and MDD share certain disturbed biochemical pathways, such as immunoinflammatory

control and neurotrophic support, which are understood to lead to neurological damage(MENDES-SILVA et al., 2016).

According to the World Health Organization, the main population that is affected by depression are those between 55 and 74 years old: about 6.5% of the population in this age group is affected by the disease(“WHO | Depression and Other Common Mental Disorders”, 2017). The progression of brain aging throughout life involves cumulative changes in gene expression, though susceptibility to mental illness depends on genetic variations, each individual's molecular context, and environmental factors (SIBILLE, 2013). The term late-life depression (LLD) often refers to depressive episodes that occur around 60 years of age or later. The first episode can occur in adulthood -- which is considered early-onset depression -- or during a later age -- considered late-onset depression (GRAYSON; THOMAS, 2013; SACHS-ERICSSON et al., 2013). Thus LLD can be described as a heterogeneous and complex neuropsychiatric syndrome, which the most important features are a significant impairment of cognitive function and executive dysfunction(BUTTERS et al., 2000, 2004; RAJTAR-ZEMBATY et al., 2017).

One of the explanations for the onset of LLD is the vascular hypothesis, in which cerebrovascular diseases and their risk factors, and/or an increased accumulation of silent cerebral vascular lesions with a consequent increase in white matter may affect various biological mechanisms. These disruptions contribute to the emergence and permanence of LLD, a consequence of damage to brain circuits(ALEXOPOULOS, 1997). However, this hypothesis alone does not fully explain LLD, mainly because the chances that various diseases will affect the elderly, such as cardiovascular problems and comorbidities, are not well-defined and can increase the risk of developing LLD. The side effects that stem from treating such diseases can also increase risk; though again, older populations seem equally vulnerable to presenting side effects. As such, vascular depression may be considered a subtype of LLD, although further studies are needed to establish its mechanisms due to the poor understanding of the biochemical and structural changes in the brain that may link the hypothesis of vascular depression with LLD(AIZENSTEIN et al., 2016).

An investigation of blood plasma immunoassays of elderly patients to discover differentially expressed peripheral proteins has led to an association between LLD and inflammatory processes and reduced neurotrophic support, as well as proteostasis markers and nutrient detection(DINIZ et al., 2016). Some of these same

markers suggest that homeostatic dysregulation in patients with LLD can accelerate aging processes, which increases the risk of Alzheimer's and dementia(DINIZ et al., 2013, 2015). Another important investigation correlated a higher SASP index (a set of proteins secreted by different senescent fibroblasts) to be responsible for inducing the aging of nearby cells(COPPÉ et al., 2008; DINIZ et al., 2017) in patients with depression, and may give clues about the molecular mechanisms that culminate in LLD (COPPÉ et al., 2008; DINIZ et al., 2017). However, there are still few studies investigating the molecular mechanisms behind these processes, a line of research that could obtain a consistent biological signature of the disease.

As the pathophysiological mechanisms of LLD are distinct from major depression(GROENEWEG-KOOLHOVEN et al., 2017; WU et al., 2018), an integrated view of the biological processes and biochemical pathways altered in the disorder may contribute to the diagnosis, follow-up of treatment response, or even drug choice. Proteomics based on mass spectrometry is a tool with the potential to meet this demand. Through mass spectrometry-based proteomics, a panel of biomarker proteins that indicate the onset of the disease can be proposed and later confirmed, resulting from the investigation of the blood plasma of the elderly with depression. Additionally, altered biochemical pathways may give clues about the cascades and molecular mechanisms linked to the processes that lead the elderly to develop clinical depression. Here, we identified potential LLD-related proteins and biochemical pathways related to LLD, which will allow the investigation of the pathogenic processes that lead to the disorder.

2. Materials and methods

2.1 Subject recruitment

Exclusion criteria encompassed neurological disorder, history of psychosis, bipolar disorder, significant head trauma, and substance abuse within the past year. All participants signed an informed consent form and the university's institutional ethics committee approved the study methods. After clinical assessment, fifty individuals aged ≥ 55 years old, 19 adults with LLD and 31 adults without any psychiatric disorder, none of which had received antidepressant treatment, had their whole-blood collected in EDTA tubes by antecubital venipuncture. Plasma samples were separated,

aliquoted and stored at -80ºC. All of the samples were collected at the Medical School of the University of Minas Gerais.

2.2 Participant demographics

The participants' characteristics, reported on an outpatient basis, are presented in Table 2. Controls ranged between 60-87 years old while patients ranged between 64-90 years old. The informed generalized anxiety disorder (GAD) range was between 0-21 for patients and 0-5 for controls. The first depression episode in LLD patients occurred, at the earliest, at 18 years old and, at the latest, at 82 years old. Fifteen LLD patients presented cognitive impairment.

Table 2: Participant baseline characteristics

	Controls	Patients	p[a]
N	31	19	
Year Education, mean (SD)	8.2±5.77	4.6±3.68	0.019
Age, mean (SD)	70.3±6.39	74.0±6.91	0.061
HDRS-21[b], mean (SD)	0.9±1.33	18.7±5.19	<0.001
GAD-7[c], mean (SD)	1.0±1.5	8.7±6.29	<0.001
DRS[d], mean (SD)	132.9±7.78	114.9±16.91	0.001
Number of medical comorbidities, mean (SD)	2.6±1.55	3.0±1.51	0.374

[a] p-value for patients v controls using t-test for equality of means. [b] HDRS-21: Hamilton Depression Rating Scale 21-item version [c] GAD-7: Generalized Anxiety Disorder -7 score [d] Mattis Dementia Rating Scale – DRS

2.3 Immunoaffinity chromatography and tryptic digestion

A low-abundance fraction of plasma proteins was obtained by immunoaffinity chromatography using a 4.6mm x 100mm MARS Hu14 Immunodepletion System column (Agilent Technologies) installed on a Waters HPLC system (Waters GmbH). In this step, 40µL of plasma was added to 120 µL phosphate buffer (pH 7.4) that was prepared with 2.5% (v/v) phosphate buffer solution, 1M (Sigma-Aldrich), 10.0% (v/v) NaCl, 5M, and 0.02% (m/v) sodium azide. The phosphate buffer was also used to carry the sample into the column, according to the manufacturer’s protocol. Then, urea acidic buffer, prepared using 2.0M urea, 0.5M

glycine in water, pH 2.25, adjusted with HCl, was used to elute the high-abundance proteins bound to the column, allowing the loading of the next sample.

The samples were desalted using Amicon Ultra-4 (Merck) cartridges, allowing buffer exchange to 50 mM ammonium bicarbonate. The samples were heated with 0,2% *Rapi*Gest SF® (15 minutes, at 80ºC). Then, the proteins were reduced with dithiothreitol (100 mM, 60ºC, 30 min) and alkylated with iodoacetamide (300 mM, 30 min, room temperature, in the dark). Proteins were then digested into peptides with trypsin (Promega) at a ratio of 1:100 (w/w trypsin/protein) for 16 h at 37ºC. Digestion was quenched with 5% trifluoroacetic acid for 90 minutes at 37ºC. The samples were then centrifuged at 20,800 x*g* at 6°C for 30 min. The supernatant was recovered and the pH was adjusted using 0.5µL of 1M ammonium hydroxide before analysis.

2.4 Mass spectrometry analysis

Each sample was subjected to two-dimensional liquid chromatography. Three discontinuous acetonitrile gradients were used to perform the first-dimension chromatography step with a 2D-RP / RP Acquity UPLC, M-Class system directly coupled online to a Synapt G2-S*i* mass spectrometer (Waters Corporation). The second separation column was programmed to use a continuous 7 to 85% acetonitrile gradient over 54 minutes. The spectrometer operated in resolution mode using ion mobility separation (UDMS^{E}), peptides were injected using an electrospray ionization source in positive mode, and data-independent acquisition (DIA) was used. Details of the 2DLC-UDMS^{E} method optimization we employed can be found in Cassoli et al. 2017(S CASSOLI et al., 2017).

2.5 Data processing, statistical treatment and systems biology tools

Progenesis QI for Proteomics software (version 4.0) was used to process the spectra and normalize their abundances. Quantification was performed using the Hi-N (Hi3) method, and the parameters used for identification report were that digestion was performed with trypsin, with a maximum of one lost cleavage. Methionine oxidation was selected as a variable modification and cysteine carbamidomethylation was selected as a fixed modification. The false discovery rate (FDR) cutoff was 1%, calculated using a reverse sequence database generated on-the-fly by Progenesis. The established mass error cutoff was 20ppm. Ion matching requirements considered proteins that had at least 2 fragments per peptide, 5 fragments per protein, and 1

peptide per protein. The search was performed against the Swiss-Prot Human Proteomic Database(BOUTET et al., 2007) (available version of November 11, 2019).

After keratin and remaining 14 high-abundant proteins and their isoforms, and considering only proteins identified in at least 70% of all samples (control subjects and patients), IBM(®) SPSS(®) statistics 20.0 (Armonk, NY: IBM Corp.) was used to carry out statistical analyses, using Pearson correlation and independent T-test with Benjamini-Hochberg adjusted p-value (FDR 5%). These analyses were used to generate a potential panel of proteins related to cognitive impairment, early onset of symptoms, recurrent episodes, and Hamilton Rating Scale for Depression (HDRS) score, used to assess patients' levels of depression. Then, biological network gene ontology tool BiNGO (MAERE; HEYMANS; KUIPER, 2005) via Cytoscape(SHANNON et al., 2003) was used to explore the biological processes and molecular functions of annotated genes related to proteins differentially expressed in LLD, applying a hypergeometric test with Benjamini & Hochberg FDR correction. Cytoscape StringApp (DONCHEVA et al., 2019) was applied to investigate the interactions between differentially expressed proteins between control individuals and patients and Metascape (ZHOU et al., 2019) was used to investigate the pathways and process enrichment analysis between subgroups of LLD patients.

3. Results

3.1 Protein Profiling for LLD samples

Spectra processing reported 12,136 peptides, from which 594 proteins were identified. After exclusion of keratin, remaining 14 high-abundant proteins and their isoforms, and considering only proteins identified in at least 70% of all samples, 335 remaining proteins were selected for system biology analysis (Supplementary table 1).

Independent t-tests ($p<0.05$), with p-values adjusted by the Benjamini-Hochberg method (FDR 5%), revealed 96 proteins which are suggested to be significantly differentially expressed (Table 3). These 96 proteins are associated with 69 biological processes and 24 molecular functions (Supplementary table 2). The top biological processes affected by these altered proteins include processes linked to acute inflammatory response, response to wounding, complement activation, activation of plasma proteins involved in the acute inflammatory response, and humoral immune response (Fig. 10A). The top molecular functions associated with

these altered proteins are related to endopeptidase inhibitor and regulator activity, peptidase inhibitor and regulator activity, and enzyme regulatory activity (Fig. 10B). The interaction between significantly different proteins is shown in Fig. 11. The top 5 pathways affected by those proteins are intrinsic pathway of fibrin clot formation, complement cascade, formation of fibrin clot, regulation of IGF transport and uptake by IGFBPs, and regulation of complement cascade, and the location of proteins presented in Figure 11 is mainly extracellular space**.**

Table 3: 96 proteins suggested to be significantly differentially expressed between LLD patients and controls.

Gene Name	Protein Name	Accession	P.value	FDR 5%	Log2 Fold Change
TLE4	Transducin-like enhancer protein 4	Q04727	4.36E-06	1.83E-04	-5.596
SAA1	Serum amyloid A-1 protein	P0DJI8	0.00979	4.00E-02	2.317
LILRA1	Leukocyte immunoglobulin-like receptor subfamily A member 1	O75019	3.43E-04	3.19E-03	2.274
DYRK1A	Dual specificity tyrosine-phosphorylation-regulated kinase 1A	Q13627	0.00884	3.85E-02	-2.103
SDR42E2	Putative short-chain dehydrogenase/reductase family 42E member 2	A6NKP2	0.01120	4.17E-02	1.873
LMX1A	LIM homeobox transcription factor 1-α	Q8TE12	0.00082	6.28E-03	-1.836
MBL2	Mannose-binding protein C	P11226	0.00185	1.11E-02	1.772
MINPP1	Multiple inositol polyphosphate phosphatase 1	Q9UNW1	0.00293	1.58E-02	-1.621
UPP2	Uridine phosphorylase 2	O95045	3.03E-04	3.38E-03	-1.493
THBS1	Helicase POLQ-like	P07996	3.26E-04	3.20E-03	-1.328

NKX6-2	Homeobox protein Nkx-6.2	Q9C056	3.30E-04	3.16E-03	-1.320
SNHG28	Putative uncharacterized protein SNHG28	P0DPA3	0.01324	4.81E-02	1.262
CACNA1C	Voltage-dependent L-type calcium channel subunit α-1C	Q13936	0.00138	8.72E-03	-1.187
SERPINA7	Thyroxine-binding globulin	P05543	0.00210	1.23E-02	-1.170
CFAP47	Cilia- and flagella-associated protein 47	Q6ZTR5	0.01092	4.15E-02	1.149
HBE1	Hemoglobin subunit epsilon	P02100	2.78E-09	9.31E-07	1.067
JCHAIN	Immunoglobulin J chain	P01591	0.00105	7.65E-03	1.000
EED	Polycomb protein EED	O75530	4.19E-07	4.68E-05	-0.932
FAM47DP	Putative protein FAM47D	A6NHR8	3.88E-05	7.60E-04	0.920
POSTN	Periostin	Q15063	0.00086	6.42E-03	0.893
GP1BA	Platelet glycoprotein Ib α chain	P07359	2.68E-05	6.00E-04	0.855
GRM5	Metabotropic glutamate receptor 5	P41594	0.01405	4.92E-02	-0.850
FAM171A1	Protein FAM171A1	Q5VUB5	2.57E-05	6.60E-04	0.850
IGF2	Insulin-like growth factor II	P01344	0.01056	4.08E-02	-0.847
CD1E	T-cell surface glycoprotein CD1e_ membrane-associated	P15812	0.00873	3.85E-02	0.827
HYI	Putative hydroxypyruvate isomerase	Q5T013	0.00940	3.94E-02	-0.806
INTS4	Integrator complex subunit 4	Q96HW7	2.37E-04	2.94E-03	0.752
C11orf49	UPF0705 protein C11orf49	Q9H6J7	4.04E-04	3.56E-03	-0.730
C1QA	Complement C1q subcomponent subunit A	P02745	3.25E-04	3.30E-03	-0.713
SERPINC1	Antithrombin-III	P01008	2.01E-04	2.93E-03	-0.682

APCS	Serum amyloid P-component	P02743	7.99E-05	1.41E-03	-0.676
CDH1	Cadherin-1	P12830	0.00081	6.33E-03	0.673
ITGAE	Integrin α-E	P38570	3.11E-04	3.26E-03	0.662
C7	Complement component C7	P10643	0.00118	8.41E-03	0.649
TMEM102	Transmembrane protein 102	Q8N9M5	5.11E-06	1.90E-04	0.644
FETUB	Fetuin-B	Q9UGM5	0.00152	9.43E-03	-0.635
C2	Complement C2	P06681	0.01112	4.18E-02	0.633
IGFALS	Insulin-like growth factor-binding protein complex acid labile subunit	P35858	0.00060	5.05E-03	-0.621
HELQ	Thrombospondin-1	Q8TDG4	2.77E-04	3.20E-03	0.619
ADAMTS12	A disintegrin and metalloproteinase with thrombospondin motifs 12	P58397	0.00244	1.41E-02	0.609
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	Q12805	2.74E-05	5.70E-04	-0.598
FCN3	Ficolin-3	O75636	0.00553	2.65E-02	0.566
SERPINA10	Protein Z-dependent protease inhibitor	Q9UK55	4.20E-04	3.61E-03	-0.561
CDC20B	Cell division cycle protein 20 homolog B	Q86Y33	0.00840	3.75E-02	0.544
PKN1	Serine/threonine-protein kinase N1	Q16512	0.00499	2.50E-02	-0.541
C4A	Complement C4-A	P0C0L4	4.27E-05	7.90E-04	-0.525
AMBP	Protein AMBP	P02760	0.00250	1.42E-02	-0.515
F10	Coagulation factor X	P00742	0.00637	2.96E-02	-0.513
F9	Coagulation factor IX	P00740	4.67E-07	3.91E-05	0.504
KHSRP	Far upstream element-binding protein 2	Q92945	0.00889	3.77E-02	-0.495

CPB2	Carboxypeptidase B2	Q96IY4	2.62E-05	6.30E-04	0.489
GAPDHS	Glyceraldehyde-3-phosphate dehydrogenase_ testis-specific	O14556	0.01331	4.79E-02	-0.478
PON3	Serum paraoxonase/lactonase 3	Q15166	3.77E-04	3.41E-03	0.478
CEP104	Centrosomal protein of 104 kDa	O60308	0.00134	8.80E-03	0.467
F11	Coagulation factor XI	P03951	8.93E-05	1.50E-03	-0.453
SHBG	Sex hormone-binding globulin	P04278	0.00074	5.86E-03	0.444
VILL	Villin-like protein	O15195	3.07E-04	3.32E-03	-0.443
CP	Ceruloplasmin	P00450	0.00403	2.08E-02	-0.438
TBL2	Transducin beta-like protein 2	Q9Y4P3	2.53E-07	4.24E-05	-0.432
IGHV3-30-3	Immunoglobulin heavy variable 3-30-3	P0DP02	1.33E-06	7.43E-05	-0.420
KLKB1	Plasma kallikrein	P03952	7.59E-07	5.09E-05	-0.416
	Immunoglobulin kappa light chain	P0DOX7	2.36E-04	3.04E-03	-0.398
DRP2	Dystrophin-related protein 2	Q13474	0.00272	1.52E-02	-0.397
LBP	Lipopolysaccharide-binding protein	P18428	0.01043	4.05E-02	-0.389
SERPINA3	α-1-antichymotrypsin	P01011	5.19E-06	1.74E-04	0.388
PIK3C2B	Phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit β	O00750	0.00889	3.82E-02	-0.386
BCHE	Cholinesterase	P06276	0.00119	8.31E-03	-0.375
WFDC2	WAP four-disulfide core domain protein 2	Q14508	7.01E-06	2.14E-04	-0.375
C1RL	Complement C1r subcomponent-like protein	Q9NZP8	0.00161	9.81E-03	-0.370

HPX	Hemopexin	P02790	9.81E-06	2.74E-04	0.369
CNDP1	Beta-Ala-His dipeptidase	Q96KN2	2.07E-04	2.89E-03	0.349
MASP2	Mannan-binding lectin serine protease 2	O00187	0.00123	8.24E-03	-0.340
C1S	Complement C1s subcomponent	P09871	0.01021	4.02E-02	0.334
MIA3	Transport and Golgi organization protein 1 homolog	Q5JRA6	0.01405	4.94E-02	0.331
LTF	Lactotransferrin	P02788	1.52E-04	2.42E-03	-0.328
ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2	P19823	1.57E-04	2.39E-03	-0.321
CFP	Properdin	P27918	0.00137	8.83E-03	-0.304
FCHO2	F-BAR domain only protein 2	Q0JRZ9	2.75E-04	3.29E-03	-0.300
MMP17	Matrix metalloproteinase-17	Q9ULZ9	0.00979	4.05E-02	0.283
FUBP1	Far upstream element-binding protein 1	Q96AE4	0.00066	5.36E-03	-0.281
HSP90AB1	Heat shock protein HSP 90-beta	P08238	0.00512	2.52E-02	-0.269
PROS1	Vitamin K-dependent protein S	P07225	1.83E-06	8.76E-05	0.267
CA1	Carbonic anhydrase 1	P00915	0.01288	4.75E-02	-0.263
GC	Vitamin D-binding protein	P02774	2.13E-04	2.85E-03	-0.261
USP40	Ubiquitin carboxyl-terminal hydrolase 40	Q9NVE5	0.00294	1.56E-02	0.258
ATP8A1	Phospholipid-transporting ATPase IA	Q9Y2Q0	0.00274	1.51E-02	0.243
NEFM	Neurofilament medium polypeptide	P07197	0.00460	2.34E-02	-0.238
PON1	Serum paraoxonase/arylesterase 1	P27169	0.01356	4.85E-02	0.228

CFI	Complement factor I	P05156	0.00768	3.48E-02	-0.224
APOC3	Apolipoprotein C-III	P02656	0.00121	8.27E-03	-0.216
ITIH4	Inter-alpha-trypsin inhibitor heavy chain H4	Q14624	0.00992	4.00E-02	-0.214
ITIH	Inter-alpha-trypsin inhibitor heavy chain H1	P19827	0.01007	4.03E-02	0.210
KNG1	Kininogen-1	P01042	0.00324	1.70E-02	0.192
PPP1R26	Protein phosphatase 1 regulatory subunit 26	Q5T8A7	0.00610	2.88E-02	-0.190
CUL2	Cullin-2	Q13617	0.00747	3.43E-02	-0.171
IGFBP3	Insulin-like growth factor-binding protein 3	P17936	0.00533	2.59E-02	-0.146

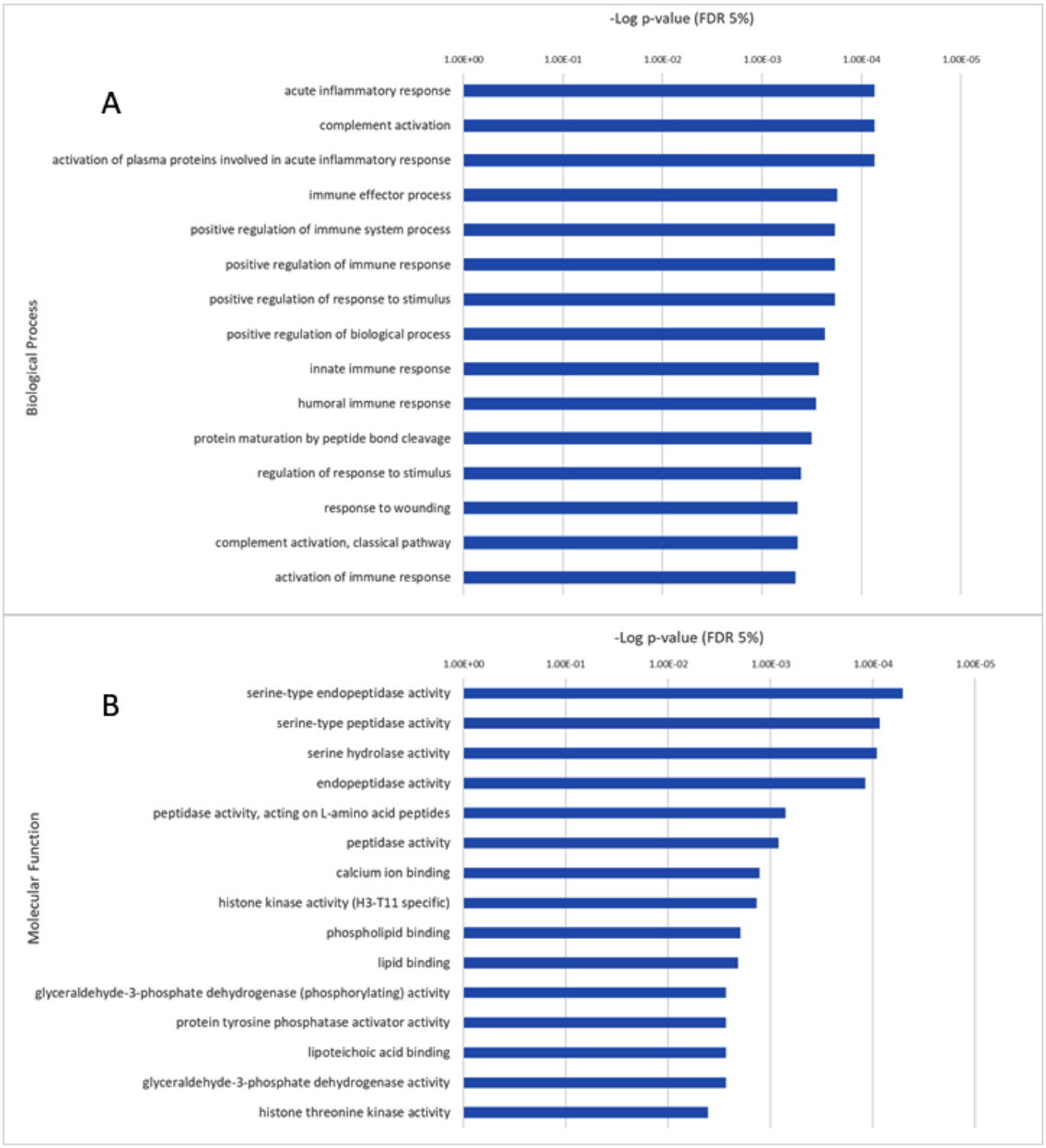


Figure 10: A) Top 15 biological processes affected by altered proteins in LLD. B) Top 15 molecular functions affected by altered proteins in LLD.

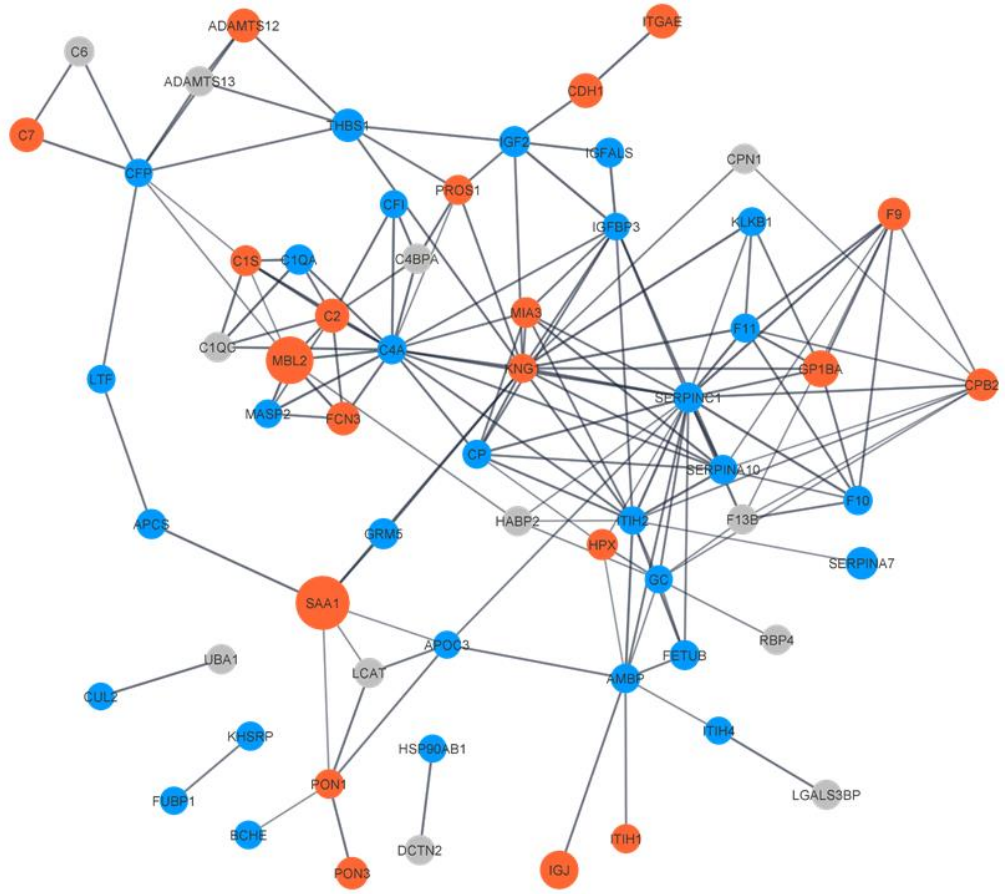


Figure 11. Interaction net of proteins suggested to be differentially expressed found in LLD. Red dots show upregulated proteins (p-value <0.05, FDR 5%) and blue dots, downregulated proteins. Grey dots represent proteins which p-value <0.1. The size of the dots is related to their ratio to control individuals mean regulation.

3.2 Protein expressions in LLD patients affected by cognitive impairment and recurrent episodes of LLD

Comparing two subgroups of LLD patients, those with cognitive impairment and those with recurrent episodes of LLD, with LLD patients without those traits, and considering unadjusted significant p-values, 14 proteins were highlighted by their p-value and which may be associated with cognitive decline (Supplementary table 3). Ten proteins were highlighted by their uncorrected p-value that may be associated with recurrent episodes (Supplementary table 4).

We decide to proceed with the investigation of proteins with uncorrected p-value by considering the probability of those proteins may reveal a clue about different traits in LLD. An analysis by Metascape tool of gene list composed by 96 LLD profiling

proteins, 14 proteins which may be altered in patients with cognitive impairment, and 10 proteins which may be altered in patients with recurrent episodes shows an overlap based the shared term level, despite the overlap between the gene shared between these groups are low (Fig.12). Thus, the results suggest that the immune system may be one of the biochemical pathways involved with cognitive impairment, in particular intrinsic pathway of fibrin clot formation, as well as the complement and coagulation cascades may play a role in recurrent episodes of depression in elderly (Fig. 13).

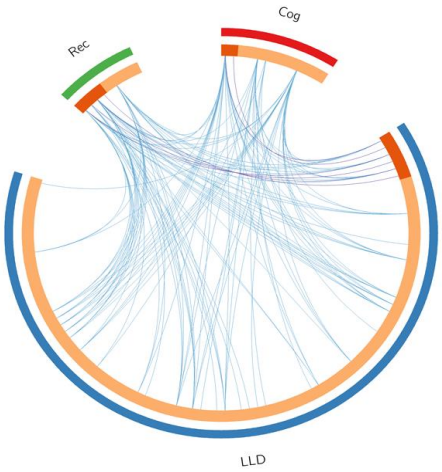


Figure 12. Considering enriched ontology terms, blue curves link the genes (the inner circle in dark orange) that share function or pathways.

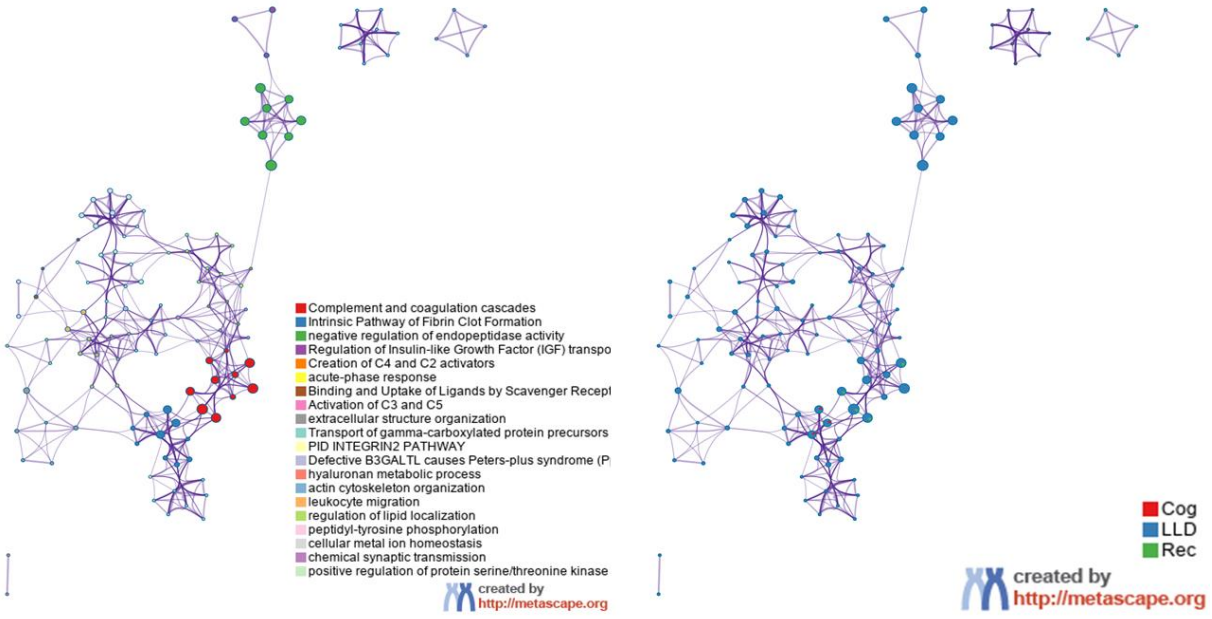
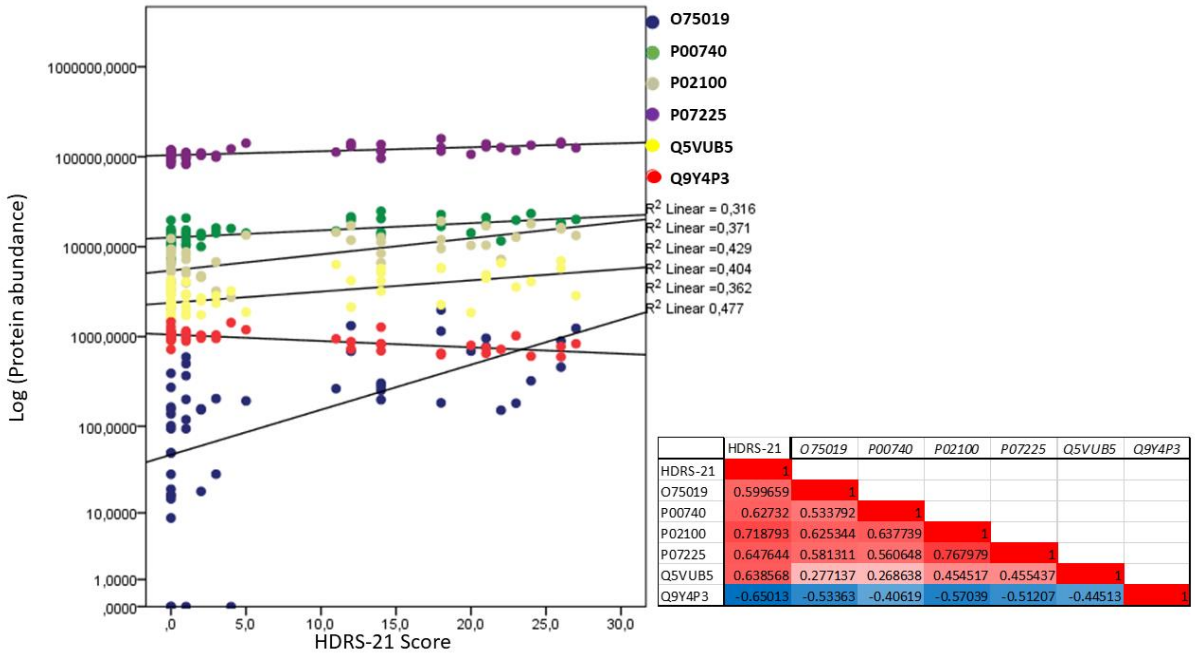


Figure 13. (A) Network of enriched terms between protein profiling of LLD and the subgroups of patients. (B) Network of enriched terms represented as pie chart, based on the identities of the gene lists of three groups.

3.4 Correlation between protein profile and HDRS

A Pearson correlation test was performed using the HDRS scores of LLD patients and controls. Considering a significance level of $p<0.05$, a moderate to strong correlation ($0.600 < r < 0.719$) was found between the HDRS score and 6 proteins in LLD patients (Fig. 14). An increased presence of these 5 proteins and decrease of 1 protein in plasma suggest that a higher HDRS score may be related to alterations in coagulation process - considering the proteins P00740 (coagulation factor IX precursor, gene name F9) and P07225 (vitamin K-dependent protein S precursor, gene name PROS1). These proteins also may be part of hemostasis pathway, as well as P02100 (Hemoglobin subunit epsilon, gene name HBE1). The protein O75019 (leukocyte immunoglobulin-like receptor subfamily A, gene name LILRA1) plays a role in immunoregulatory interactions between a lymphoid and a non-lymphoid cell, and the proteins Q5VUB5 (protein FAM171A1) and Q9Y4P3 (transducin beta-like protein 2, gene name TBL2) were not associated to a specific biological process.



	HDRS-21	O75019	P00740	P02100	P07225	Q5VUB5	Q9Y4P3
HDRS-21	1						
O75019	0.599659	1					
P00740	0.62732	0.533792	1				
P02100	0.718793	0.625344	0.637739	1			
P07225	0.647644	0.581311	0.560648	0.767979	1		
Q5VUB5	0.638568	0.277137	0.268638	0.454517	0.455437	1	
Q9Y4P3	-0.65013	-0.53363	-0.40619	-0.57039	-0.51207	-0.44513	1

Figure 14. Correlation between the HDRS score and 6 proteins in LLD patients

Discussion

Aging is a major risk factor for many serious diseases, such as cancer, cardiovascular disease, metabolic and neurodegenerative diseases; the dynamics of

interactions between the different biochemical pathways affected by aging that may be related to late-life depression (LLD) are not yet fully known (ARMANIOS et al., 2015; RUTHERFORD et al., 2017). Like many other diseases, LLD may have genetic components, and, in that case, CNS genetic alterations might be displayed in peripheral tissues (SULLIVAN; FAN; PEROU, 2006). Moreover, blood plasma may carry residual sub-proteomes of all the body tissues. For this reason, we aimed to investigate whether differences in protein profiling exist between elderly subjects with LLD and control ones. Additionally, an investigation was performed to compare subgroups of patients with LLD to determine if there are any molecular characteristics that may correspond to the different symptoms presented by these patients.

We found 96 proteins which may be differentially expressed and may that confirm an association between LLD and an overall upregulation of complement cascade; but we also found downregulated pathways. These include the innate immune system, specifically, the classical antibody-based complement activation and activation of C3 and C5, whose presence may indicate the activation of alternative pathway; and a significant upregulation of pathways that lead to UNC93B1 deficiency, associated with toll-like receptor (TLR) signaling cascade. Complement system alterations have been associated with LLD and other neuropsychiatric disorders (ISHII et al., 2018; SHIN et al., 2019), and regulation of TLR has been associated with an antidepressant response, which may be revealed as a potential target of new drugs (HUNG, 2018).

The results of this study indicate that patients with LLD exhibit changes in biological processes related to the inflammatory and immune systems, metabolic and endocrine pathways, blood coagulation pathways, and post-translational protein modifications. Pathways related to vesicle-mediated transport, such as binding and uptake of ligands by scavenger receptors, and pathways related to neuronal systems, such as neurexins and neuroligins, might be altered as well. Vesicle-mediated transport may play a role in forms of synaptic plasticity and may have relevance in psychiatric disorders (WANG, 2008). Neurexins and neuroligins are presynaptic(LI et al., 2015) and postsynaptic(DANG et al., 2018) adhesion molecules, respectively, and may suggest an alteration in synaptic plasticity which may increase the depression and stress susceptibility (HESHMATI et al., 2018). The altered proteins related to these pathways are also associated with disorders such as Alzheimer's disease (SHIBATA et al., 2013), postmenopausal changes (GAMBERA et al., 2004; KORSE et al., 2009),

cardiovascular disease (MITRA et al., 2018; SIMON et al., 1997), and some cancers (KASPER et al., 2008; SIMONIAN et al., 2018). Therefore, changes in these pathways are related to processes that also occur during the aging process, strengthening the possibility that they may be also associated with the biochemical changes that lead to depression in the elderly.

Hemostasis pathways are upregulated in LLD patients compared to control subjects, except for the common pathway of fibrin clot formation. A prothrombotic phenotype has been associated with MDD patients (LOPEZ-VILCHEZ et al., 2014), as well as has been proposed as a biomarker for suicidal behavior in MDD (YANG et al., 2016). Also, depression can be associated with a higher risk of cardiovascular disease (PANAGIOTAKOS et al., 2004) and may negatively impact cardiovascular patients outcomes (SHIMBO et al., 2005). Therefore, there may be associations, though still unclear, between thrombogenicity, an increase in cardiovascular risk, and depression (CHAUDHARY et al., 2016; SINGH et al., 2015; SWIERINGA et al., 2018).

Alterations found in the metabolism of proteins included a downregulation of post-translational protein phosphorylation and decreased regulation of insulin-like growth factor (IGF) transport and uptake by IGF binding proteins. However, there is an upregulation of gamma-carboxylation hypusine formation and arylsulfatase activation. In older adults, the reduction of IGF-1 levels has been associated with a higher risk of developing depression (CHIGOGORA et al., 2016; MUELLER et al., 2018; VAN VARSSEVELD et al., 2015). A deficiency of arylsulfatase A, a lysosomal enzyme involved in the degradation of glycolipids, leads to a severe genetic disorder that affects myelin sheaths (VIRGENS et al., 2015) and may be implicated in major depression (RAYMER; WATERS; PRICE, 2005; RICKETTS et al., 1996).

We found correlations between the presence of 6 proteins and the overall score of HDRS, including health subjects. One protein, coagulation factor IX (F9), is a vitamin k-dependent glycoprotein. The vitamin k-dependent protein S (PROS1) was also found to be upregulated in LLD patients and has a correlated increased presence in plasma of patients with higher score of HDRS. PROS1 is central to the protein C pathway, a regulatory system of blood coagulation pathways. Coagulation factors have been associated with depressive symptoms in a study with a large cohort of young individuals with some symptoms of depression, though otherwise healthy (DOULALAS et al., 2006). Coagulation factors were also found to be altered in a study investigating serum proteins associated with depression (WANG et al., 2016). In one study involving

five thrombotic patients with psychosis undergoing treatment with the anticoagulant warfarin, a normalization of plasminogen activation levels may have induced remission in patients with symptoms of psychosis (HOIRISCH-CLAPAUCH; NARDI, 2013). Unfortunately, there is still a lack of studies involving coagulation factor 9 in elderly patients, which could give clues about the mechanism of this factor in LLD. Considering the inflammatory component of this disease, the investigation of individual clotting factors would certainly help to elucidate the mechanism that links depression and aging.

Overall, these findings confirm that LLD has a heterogeneous pathophysiology, with different pathways contributing to distinct phenotypes. Some of the limitations of this study are the small sample number and the reduced statistical power by unequal group sizes. We recognize that further studies are needed with a larger cohort for a better molecular design of the conditions coexisting in LLD. Also, as common in projects involving proteomics, there is a great number of variables to deal with. The correlations and biochemical pathways revealed here, though they may not directly indicate how these relationships can be explored to improve the quality of life of these patients, they can point the way for future research.

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Conflict of Interest Statement

The authors declare no conflict of interest.

8. CONCLUSÃO

Nesta dissertação, exploramos o perfil proteômico relativo de pacientes com LLD, com o propósito de ajudar a elucidar a depressão em pessoas idosas, além de identificar de vias bioquímicas relacionadas à doença. Este estudo, apesar de exploratório, abre caminhos para muitas discussões envolvendo diversos aspectos da depressão geriátrica, e algumas características comuns a subgrupos de indivíduos afetados por esta doença ainda não se encontram totalmente elucidadas. A depressão geriátrica, como muitas doenças psiquiátricas, também é uma doença relacionada a neuroinflamação, e neste estudo ela revela um envolvimento significativo do sistema imune inato, em especial do sistema complemento. A ativação de processos inflamatórios não é só associada a depressão em adultos jovens, mas também pode integrar a etiologia da depressão em pessoas idosas. Apesar de a depressão maior e a depressão geriátrica possuir características em comuns, outras características únicas apresentadas por pacientes com depressão geriátrica devem ser consideradas (RIDDLE et al., 2017).

O envelhecimento é o maior fator de risco em muitas doenças graves, e a dinâmica entre as vias bioquímicas afetadas pelo processo de envelhecimento que podem estar relacionadas à depressão geriátrica ainda não foram totalmente elucidadas (ARMANIOS et al., 2015; RUTHERFORD et al., 2017). Os resultados deste estudo indicam alterações em processos biológicos relacionados ao sistema imune e resposta inflamatória, vias metabólicas e endócrinas, vias de coagulação e modificações pós-traducionais de proteínas. Vias relacionadas ao transporte mediado por vesículas, e vias relacionadas ao sistema neuronal também estão alteradas, como neurexinas and neuroliginas. Alterações proteômicas relacionadas a essas vias estão associadas à doença de Alzheimer(SHIBATA et al., 2013), mudanças pós menopausa (GAMBERA et al., 2004; KORSE et al., 2009), doenças cardiovasculares (MITRA et al., 2018; SIMON et al., 1997) e alguns tipos de câncer(KASPER et al., 2008; SIMONIAN et al., 2018). Portanto, alterações nessas vias estão relacionadas a processos que também ocorrem durante o envelhecimento e podem ser associadas às mudanças bioquímicas que acarretam na depressão geriátrica.

Neste trabalho sugere-se que o prejuízo cognitivo presente na depressão geriátrica esteja mais significativamente associado a vias de ativação do sistema complemento, como outros estudos também já mencionaram (CERRI et al., 2009;

D’AVILA et al., 2018). Tendo em vista que a severidade da depressão, e não o prejuízo cognitivo, pode estar associado à incapacidade na depressão geriátrica (MORIN et al., 2019), esses resultados sugerem diferentes alvos para tratamentos com o propósito de prolongar a qualidade de vida em idosos com depressão geriátrica. Além disso, pacientes idosos com episódios recorrentes de depressão apresentaram alterações em vias de transmissão de sinapse química e eliminação de neurotransmissores, o que pode ser relacionado a outros achados envolvendo a disruptura da estabilidade de redes e perda de homeostase neuronais (ANDREESCU et al., 2019; D’AVILA et al., 2018).

Um dos achados mais interessantes do trabalho foi a correlação entre seis proteínas e a pontual geral na escala Hamilton, que mede a severidade da depressão. Algumas destas as proteínas que foram correlacionadas possuem papel na homeostase do cérebro. Os achados deste estudo confirmam que diferentes alterações em vias bioquímicas contribuem para diferentes fenótipos da depressão geriátrica, tornado sua patofisiologia heterogênea e cuja complexidade dificulta sua investigação sob aspectos gerais.

9. LIMITAÇÕES DO ESTUDO

Algumas das limitações deste estudo são o limitado número de amostras e o poder estatístico reduzido por tamanhos desiguais de grupos, restrições que tentamos minimizar através de testes estatísticos mais adequados além dos normalmente empregados em ensaios proteômicos. Além disso, como é comum nos trabalhos de proteômica, há um grande número de variáveis a serem tratadas e os resultados podem ser explorados posteriormente através de ferramentas computacionais, como aprendizado de máquina e modelo de intercepto randômico. As correlações e caminhos bioquímicos revelados aqui podem não indicar diretamente como essas relações podem ser exploradas para melhorar a qualidade de vida desses pacientes, mas podem apontar o caminho para futuras pesquisas.

10. PERSPECTIVAS

A depressão geriátrica afeta 4,4% da população maior de 60 anos no planeta, e com o aumento dos recursos médicos para prolongamento da vida nesta faixa etária, as perspectivas no futuro é que essa fração afetada corresponda a um número cada vez maior da população, promovendo uma sobrecarga nos sistemas de saúde, e prejuízo na produtividade de cuidadores familiares. Considerando a complexidade embutida em um grupo experimental com depressão geriátrica, a investigação dos mecanismos que levam à doença demanda ferramentas estatísticas e emprego de diferentes ciências ômicas associados com ferramentas de biologia de sistemas, com o propósito de desvendar esses processos. Este trabalho realiza uma fração desta investigação, cujos resultados deverão ser validados em um maior número de pacientes. Apesar da inexistência de biomarcadores estabelecidos para doenças psiquiátricas, os dados obtidos neste trabalho podem fornecer uma pista para o futuro estabelecimento de um teste bioquímico, que possa auxiliar no diagnóstico da depressão geriátrica por médicos e psiquiatras.

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12. ANEXOS

Supplementary table 1: Proteins expressed in LLD plasma patients								
Independent Samples T-Test								
Gene Name	Accession	Sig. (2-tailed)	Benjamini-Hochberg Adjusted P value (FDR 5%)	Mean Difference	Std. Error Difference	Lower	Upper	Log2LLD:CTRL Ratio
HBE1	P02100	2.78E-09	9.313E-07	-6,721.5082	923.7810	-8,578.8943	-4,864.122130	1.067172
F9	P00740	4.67E-07	3.911E-05	-5,453.5935	936.5738	-7,336.7013	-3,570.485687	0.503710
TBL2	Q9Y4P3	2.53E-07	4.238E-05	272.7779	45.4789	181.3364	364.219467	-0.431826
EED	O75530	4.19E-07	4.679E-05	2,927.1697	483.0259	1,950.3236	3,904.015852	-0.931800
KLKB1	P03952	7.59E-07	5.085E-05	13,857.9309	2,438.1178	8,955.7665	18,760.095271	-0.415738
IGHV3-30-3	P0DP02	1.33E-06	7.426E-05	1,784.1136	322.9698	1,134.7392	2,433.488003	-0.419856
PROS1	P07225	1.83E-06	8.758E-05	-21,469.9209	3,952.8158	-29,417.5897	-13,522.252128	0.267253
SERPINA3	P01011	5.19E-06	1.739E-04	-118,625.5550	23,127.6750	-165,126.8623	-72,124.247755	0.388106
TLE4	Q04727	4.36E-06	1.826E-04	537.2836	96.0632	341.1033	733.463913	-5.596133
TMEM102	Q8N9M5	5.11E-06	1.902E-04	-1,776.0836	345.9914	-2,471.7459	-1,080.421378	0.643556
WFDC2	Q14508	7.01E-06	2.135E-04	37,814.8492	7,501.4034	22,732.2668	52,897.431705	-0.375040
HPX	P02790	9.81E-06	2.739E-04	-170,182.5404	34,433.0789	-239,414.8856	-100,950.195239	0.369129
EFEMP1	Q12805	2.74E-05	0.00057	4,526.8447	976.1919	2,564.0793	6,489.610140	-0.597598
GP1BA	P07359	2.68E-05	0.00060	-757.7386	163.1554	-1,085.7845	-429.692735	0.854974
CPB2	Q96IY4	2.62E-05	0.00063	-9,638.6051	2,072.6623	-13,805.9720	-5,471.238132	0.489387
FAM171A1	Q5VUB5	2.57E-05	0.00066	-2,008.6253	383.7191	-2,802.1791	-1,215.071506	0.849639
FAM47DP	A6NHR8	3.88E-05	0.00076	-4,048.9313	893.3187	-5,845.0690	-2,252.793574	0.920401

C4A	P0C0L4	4.27E-05	0.00079	59,529.4662	13,217.2307	32,954.4426	86,104.489707	-0.524510
APCS	P02743	7.99E-05	0.00141	63,232.1711	14,661.5426	33,753.1639	92,711.178215	-0.676364
F11	P03951	8.93E-05	0.00150	2,247.9217	525.3995	1,191.5351	3,304.308194	-0.452552
ITIH2	P19823	1.57E-04	0.00239	51,102.9542	12,456.6770	26,057.1265	76,148.781934	-0.321371
LTF	P02788	1.52E-04	0.00242	1,965.3042	477.7036	1,004.8168	2,925.791633	-0.328359
GC	P02774	2.13E-04	0.00285	86,000.3271	21,463.8307	42,844.4031	129,156.251190	-0.260898
CNDP1	Q96KN2	2.07E-04	0.00289	-2,790.2686	694.7777	-4,187.2128	-1,393.324385	0.349390
SERPINC1	P01008	2.01E-04	0.00293	59,620.7740	14,319.9123	30,525.1902	88,716.357759	-0.681765
INTS4	Q96HW7	2.37E-04	0.00294	-3,650.6513	918.6559	-5,497.7328	-1,803.569809	0.751860
	P0DOX7	2.36E-04	0.00304	4,071.8019	1,024.2484	2,012.4124	6,131.191338	-0.397731
NKX6-2	Q9C056	3.30E-04	0.00316	428.9816	110.9095	205.9831	651.980207	-1.319829
LILRA1	O75019	3.43E-04	0.00319	-512.5327	119.0152	-760.7745	-264.290821	2.274075
HELQ	Q8TDG4	2.77E-04	0.00320	-367.9996	93.7851	-556.5672	-179.432034	0.618723
THBS1	P07996	3.26E-04	0.00320	888.8012	229.5158	427.3288	1,350.273646	-1.328098
ITGAE	P38570	3.11E-04	0.00326	-7,208.0231	1,854.2497	-10,936.2421	-3,479.804132	0.662246
FCHO2	Q0JRZ9	2.75E-04	0.00329	9,904.3190	2,522.2142	4,833.0675	14,975.570482	-0.300230
C1QA	P02745	3.25E-04	0.00330	18,559.1553	4,661.9117	9,098.6469	28,019.663673	-0.713050
VILL	O15195	3.07E-04	0.00332	199.5543	51.2800	96.4491	302.659602	-0.443255
UPP2	O95045	3.03E-04	0.00338	533.8465	137.0309	258.3274	809.365671	-1.492867
PON3	Q15166	3.77E-04	0.00341	-6,721.1269	1,757.0252	-10,253.8628	-3,188.390868	0.477597
C11orf49	Q9H6J7	4.04E-04	0.00356	1,986.5033	522.3399	936.2686	3,036.738114	-0.730172
SERPINA10	Q9UK55	4.20E-04	0.00361	3,870.0071	1,020.9453	1,817.2591	5,922.755129	-0.561068
IGFALS	P35858	0.00060	0.00505	12,532.2126	3,412.0557	5,671.8148	19,392.610390	-0.621074

FUBP1	Q96AE4	0.00066	0.00536	1,373.5220	376.8457	615.8229	2,131.221098	-0.280821
SHBG	P04278	0.00074	0.00586	-1,315.0374	364.5458	-2,048.0058	-582.068967	0.444100
LMX1A	Q8TE12	0.00082	0.00628	10,880.2209	3,034.3163	4,768.5516	16,991.890236	-1.836146
CDH1	P12830	0.00081	0.00633	-350.5146	98.0580	-547.6735	-153.355772	0.673318
POSTN	Q15063	0.00086	0.00642	-1,577.7507	443.8624	-2,470.1958	-685.305583	0.892780
JCHAIN	P01591	0.00105	0.00765	-2,715.2835	778.5843	-4,280.7322	-1,149.834732	0.999714
MASP2	O00187	0.00123	0.00824	7,636.3501	2,221.9939	3,168.7319	12,103.968298	-0.339853
APOC3	P02656	0.00121	0.00827	2,951.3755	857.4275	1,227.4021	4,675.348982	-0.215850
BCHE	P06276	0.00119	0.00831	14,212.4304	4,123.0241	5,922.5349	22,502.325931	-0.375482
C7	P10643	0.00118	0.00841	-19,762.0044	5,731.1685	-31,285.2909	-8,238.717905	0.648839
CACNA1C	Q13936	0.00138	0.00872	774.0579	227.8586	315.9175	1,232.198330	-1.186635
CEP104	O60308	0.00134	0.00880	-11,826.6439	3,471.2930	-18,806.1463	-4,847.141575	0.467382
CFP	P27918	0.00137	0.00883	2,641.5119	777.4216	1,078.4010	4,204.622877	-0.304113
FETUB	Q9UGM5	0.00152	0.00943	7,045.2249	2,094.5562	2,833.8375	11,256.612322	-0.635399
C1RL	Q9NZP8	0.00161	0.00981	1,388.7391	415.2450	553.8330	2,223.645214	-0.369773
MBL2	P11226	0.00185	0.01107	-547.5144	152.1311	-865.3275	-229.701286	1.772016
SERPINA7	P05543	0.00210	0.01234	10,702.8233	3,219.4229	4,164.5249	17,241.121629	-1.170260
ADAMTS12	P58397	0.00244	0.01409	-6,138.3518	1,918.1043	-9,994.9590	-2,281.744679	0.608705
AMBP	P02760	0.00250	0.01419	69,915.7227	21,910.6588	25,861.3906	113,970.054861	-0.514936
ATP8A1	Q9Y2Q0	0.00274	0.01505	-1,037.6143	328.5001	-1,698.1080	-377.120646	0.242775
DRP2	Q13474	0.00272	0.01519	97.6910	30.9035	35.5553	159.826691	-0.397054
USP40	Q9NVE5	0.00294	0.01563	-871.2419	277.9643	-1,430.1266	-312.357112	0.258035
MINPP1	Q9UNW1	0.00293	0.01583	699.7942	223.1881	251.0444	1,148.544006	-1.620909

KNG1	P01042	0.00324	0.01696	-31,590.4448	10,192.3312	-52,083.5001	-11,097.389542	0.192478
CP	P00450	0.00403	0.02077	119,856.4697	39,676.1709	40,082.1813	199,630.758037	-0.437883
NEFM	P07197	0.00460	0.02335	6,630.5329	2,230.4360	2,145.9407	11,115.125132	-0.238332
PKN1	Q16512	0.00499	0.02495	264.6159	89.8925	83.8748	445.356919	-0.541037
HSP90AB1	P08238	0.00512	0.02522	693.0268	236.1860	218.1429	1,167.910674	-0.268848
IGFBP3	P17936	0.00533	0.02588	693.6183	237.6253	215.8406	1,171.396117	-0.146206
FCN3	O75636	0.00553	0.02647	-4,742.8983	1,632.3178	-8,024.8932	-1,460.903400	0.566147
PPP1R26	Q5T8A7	0.00610	0.02878	1,278.6669	445.6555	382.6165	2,174.717272	-0.190147
F10	P00742	0.00637	0.02964	7,503.9053	2,630.1688	2,215.5964	12,792.214220	-0.513146
CUL2	Q13617	0.00747	0.03428	980.6819	351.0889	274.7704	1,686.593534	-0.170991
CFI	P05156	0.00768	0.03477	9,527.0137	3,423.2279	2,644.1526	16,409.874688	-0.224368
CDC20B	Q86Y33	0.00840	0.03752	-2,696.8062	981.0162	-4,669.2715	-724.340960	0.544233
KHSRP	Q92945	0.00889	0.03770	4,883.0931	1,790.4670	1,283.1180	8,483.068171	-0.495314
PIK3C2B	O00750	0.00889	0.03818	231.2450	84.7829	60.7776	401.712394	-0.385788
DYRK1A	Q13627	0.00884	0.03846	10,319.8640	3,730.1853	2,758.3194	17,881.408526	-2.103019
CD1E	P15812	0.00873	0.03848	-358.0234	130.9446	-621.3052	-94.741582	0.827416
HYI	Q5T013	0.00940	0.03936	598.9088	219.2991	155.4531	1,042.364534	-0.806426
SAA1	P0DJI8	0.00979	0.04000	-468.5674	174.1724	-818.7645	-118.370234	2.316782
ITIH4	Q14624	0.00992	0.04004	44,621.3035	16,617.8194	11,208.9382	78,033.668841	-0.213863
C1S	P09871	0.01021	0.04020	-23,936.9339	8,950.6456	-41,933.4130	-5,940.454834	0.334389
ITIH1	P19827	0.01007	0.04028	-30,787.7607	11,490.2237	-53,890.4039	-7,685.117554	0.209950
MMP17	Q9ULZ9	0.00979	0.04049	-2,678.1664	995.4130	-4,679.5783	-676.754431	0.282586
LBP	P18428	0.01043	0.04051	960.3263	360.2042	236.0873	1,684.565255	-0.389424

IGF2	P01344	0.01056	0.04082	3,040.1489	1,142.4268	743.1458	5,337.152034	-0.846698
CFAP47	Q6ZTR5	0.01092	0.04149	-304.9129	109.0098	-531.8683	-77.957498	1.149103
SDR42E2	A6NKP2	0.01120	0.04169	-1,692.8649	602.1452	-2,953.8219	-431.907851	1.872612
C2	P06681	0.01112	0.04178	-13,947.6076	5,071.8745	-24,412.3517	-3,482.863551	0.633299
CA1	P00915	0.01288	0.04749	4,563.4062	1,766.2785	1,012.0651	8,114.747178	-0.262566
GAPDHS	O14556	0.01331	0.04791	735.6358	286.1877	160.2169	1,311.054780	-0.478189
SNHG28	P0DPA3	0.01324	0.04807	-1,496.9228	560.1812	-2,652.2515	-341.594106	1.262064
PON1	P27169	0.01356	0.04847	-5,347.4447	2,086.1259	-9,541.8819	-1,153.007543	0.227865
GRM5	P41594	0.01405	0.04920	184.1969	72.2617	38.9050	329.488818	-0.850197
MIA3	Q5JRA6	0.01405	0.04937	-81.8137	32.0931	-146.3412	-17.286218	0.330999
POTEKP	Q9BYX7	0.01451	0.05008	1,427.7693	562.9396	295.9034	2,559.635173	-0.279619
CPN1	P15169	0.01478	0.05059	-1,491.9775	589.9536	-2,678.1587	-305.796313	1.635216
F13B	P05160	0.02064	0.06901	-6,576.6037	2,747.5251	-12,100.8732	-1,052.334140	0.256813
ATRN	O75882	0.02086	0.06932	3,133.2949	1,311.3863	496.5761	5,770.013781	-0.161247
ARL17A	Q8IVW1	0.02050	0.06937	-229.4089	95.7316	-421.8901	-36.927657	0.425903
LGALS3BP	Q08380	0.02168	0.07127	-817.5680	344.4948	-1,510.2212	-124.914861	0.463761
CDC42EP3	Q9UKI2	0.02206	0.07188	2,588.4987	1,093.9818	388.9010	4,788.096468	-0.242438
UBA1	P22314	0.02294	0.07376	-663.1447	269.7541	-1,224.8729	-101.416601	1.026837
C6	P13671	0.02384	0.07593	11,056.9690	4,737.8144	1,530.9548	20,582.983287	-0.195385
ADAMTS13	Q76LX8	0.02413	0.07617	-286.6447	123.0871	-534.1278	-39.161491	0.297577
FCGBP	Q9Y6R7	0.02500	0.07827	919.5697	397.4182	120.5069	1,718.632538	-0.520801
PDE4DIP	Q5VU43	0.02626	0.08158	10,586.9933	4,616.6771	1,304.5419	19,869.444640	-0.311100
MST1L	Q2TV78	0.02667	0.08206	177.5691	77.6572	21.4288	333.709308	-0.150889

MCAM	P43121	0.02712	0.08253	13,974.6366	6,130.6098	1,648.2195	26,301.053700	-0.382447
HABP2	Q14520	0.02782	0.08390	16,597.1350	7,315.5912	1,888.1530	31,306.116975	-0.163321
CFAP61	Q8NHU2	0.02832	0.08465	-240.3138	106.2823	-454.0087	-26.618956	0.443107
DCTN2	Q13561	0.03050	0.08963	-1,076.8209	482.9967	-2,047.9508	-105.690952	0.216002
MCM3AP	O60318	0.03031	0.08983	1,786.7255	800.4848	177.2428	3,396.208134	-0.902579
LCAT	P04180	0.03245	0.09357	-112.2254	50.9485	-214.6642	-9.786609	0.355984
RBP4	P02753	0.03238	0.09438	30,175.7875	13,693.9139	2,642.3283	57,709.246761	-0.185600
ACSS1	Q9NUB1	0.03379	0.09678	1,347.6692	616.7251	107.6604	2,587.678091	-0.515867
C1QC	P02747	0.03463	0.09823	7,168.7291	3,296.7545	540.1599	13,797.298184	-0.192046
C4BPA	P04003	0.03547	0.09910	35,497.0237	16,403.3853	2,515.8070	68,478.240391	-0.336226
TRPS1	Q9UHF7	0.03530	0.09937	913.7685	421.8409	65.6004	1,761.936538	-0.461186
SPTBN5	Q9NRC6	0.03699	0.10244	-1,150.0094	512.3677	-2,222.8952	-77.123613	2.135440
ACTN1	P12814	0.04076	0.11203	-460.1384	218.8287	-900.1230	-20.153795	0.255963
CFAP46	Q8IYW2	0.04199	0.11439	-7,926.5307	3,636.8537	-15,534.8980	-318.163386	2.478450
SOD3	P08294	0.04357	0.11779	-578.6343	279.1351	-1,139.8730	-17.395684	0.462871
IGLL5	B9A064	0.04687	0.12569	2,072.5883	1,015.9351	29.9139	4,115.262658	-0.601309
GNPTG	Q9UJJ9	0.04830	0.12741	86.6122	42.7420	0.6737	172.550812	-0.351419
HNRNPA2B1	P22626	0.04823	0.12815	676.1161	333.5430	5.4830	1,346.749251	-0.139031
VTN	P04004	0.05198	0.13609	15,902.8175	7,980.0960	-142.2409	31,947.875975	-0.132510
ELP2	Q6IA86	0.05370	0.13945	-273.6328	138.3450	-551.7940	4.528487	0.266106
TRIM27	P14373	0.05424	0.13967	5,235.9712	2,653.4809	-99.2097	10,571.152086	-1.198991
APOB	P04114	0.05741	0.14679	-29,643.5841	15,226.1507	-60,257.8119	970.643633	0.260820
SPTBN4	Q9H254	0.05968	0.15151	-100.1270	51.9107	-204.5005	4.246521	0.314002

CD93	Q9NPY3	0.06131	0.15440	357.9095	186.7849	-17.6467	733.465725	-0.403614
C4B	P0C0L5	0.06293	0.15609	20,764.1238	10,906.0320	-1,163.9232	42,692.170744	-0.190809
SERPINF2	P08697	0.06277	0.15700	6,476.8694	3,399.8106	-358.9078	13,312.646697	-0.169952
FCGRT	P55899	0.07395	0.17458	-18,636.3284	10,201.6264	-39,148.0731	1,875.416369	3.528143
CDH13	P55290	0.07387	0.17558	-882.1261	482.7455	-1,852.7511	88.498822	0.311154
PON2	Q15165	0.07137	0.17588	-223.0702	120.9732	-466.3031	20.162708	0.280204
NFKB1	P19838	0.07228	0.17679	1,035.8548	563.6179	-97.3749	2,169.084503	-1.516720
RPS6KB1	P23443	0.07385	0.17683	241.8203	132.3264	-24.2397	507.880301	-0.890074
CLU	P10909	0.07613	0.17704	-5,837.6110	3,220.3790	-12,312.6170	637.395084	0.154523
C1R	P00736	0.07570	0.17734	6,447.4348	3,551.3976	-693.1287	13,587.998235	-0.130774
PSKH2	Q96QS6	0.07305	0.17745	-880.6970	480.5470	-1,846.9014	85.507395	0.254961
MYH15	Q9Y2K3	0.07378	0.17786	-1,629.0816	868.6641	-3,428.3039	170.140628	1.560814
SELENOP	P49908	0.07882	0.18206	268.4581	149.4928	-32.1173	569.033489	-0.346885
F2	P00734	0.08266	0.18719	18,983.8138	10,710.2648	-2,550.6169	40,518.244352	-0.114000
C4BPB	P20851	0.08235	0.18755	-2,776.8114	1,564.9616	-5,923.3775	369.754804	0.432359
ITIH3	Q06033	0.08201	0.18815	3,188.8366	1,795.1236	-420.5012	6,798.174497	-0.134462
CRP	P02741	0.08588	0.19313	-1,708.9899	974.5599	-3,668.4738	250.494115	0.478973
HBD	P02042	0.08659	0.19341	26.4786	15.1347	-3.9517	56.908867	-0.421940
VIM	P08670	0.08734	0.19368	-167.1868	95.7942	-359.7939	25.420237	0.245223
SERPINA4	P29622	0.09216	0.20188	966.7141	562.5517	-164.3718	2,097.800042	-0.078489
ORM2	P19652	0.09160	0.20188	-149.1174	86.6213	-323.2812	25.046360	2.296837
ACTBL2	Q562R1	0.09396	0.20448	-328.9647	192.5191	-716.0502	58.120819	0.134665
ANXA9	O76027	0.09476	0.20489	-178.8131	104.9075	-389.7437	32.117532	1.949692

CFH	P08603	0.09568	0.20551	32,650.4227	19,210.8342	-5,975.5482	71,276.393611	-0.134669
ADIPOQ	Q15848	0.09705	0.20719	-776.7343	458.9520	-1,699.5192	146.050629	0.386288
HMG20A	Q9NP66	0.10215	0.21627	-50.3183	30.1958	-111.0310	10.394399	2.659859
PXDNL	A1KZ92	0.10263	0.21701	61,940.7591	37,224.1639	-12,903.4387	136,784.956927	-1.775514
FMOD	Q06828	0.10359	0.21775	28,786.8240	17,349.3851	-6,096.4528	63,670.100723	-0.927994
TFAM	Q00059	0.10830	0.22472	1,427.0611	872.0866	-326.3864	3,180.508711	-0.348798
VPS13C	Q709C8	0.11881	0.24608	3,917.6897	2,466.7396	-1,042.0227	8,877.401994	-0.131042
C8B	P07358	0.12178	0.25074	6,280.8771	3,987.3268	-1,736.1808	14,297.934997	-0.119880
S100A8	P05109	0.12387	0.25329	-296.4672	189.2911	-677.0626	84.128141	0.177324
PROZ	P22891	0.12755	0.25988	211.7413	136.5467	-62.8043	486.286890	-0.154939
HBB	P68871	0.12949	0.26033	8,345.1550	5,409.6919	-2,531.7595	19,222.069552	-0.294325
AGT	P01019	0.13144	0.26278	9,486.0530	6,181.3044	-2,942.2926	21,914.398541	-0.132939
PCOLCE	Q15113	0.13206	0.26321	-2,182.7128	1,424.6539	-5,047.1714	681.745745	0.178933
HGFAC	Q04756	0.13336	0.26364	-863.1224	565.3014	-1,999.7370	273.492151	0.102684
IGKC	P01834	0.13577	0.26800	6,340.5228	4,179.0052	-2,061.9304	14,742.975896	-0.231367
SERPIND1	P05546	0.13776	0.27035	8,569.4863	5,677.5484	-2,845.9898	19,984.962323	-0.128383
QSOX1	O00391	0.13960	0.27267	1,387.0041	923.3279	-469.4710	3,243.479173	-0.276953
ECM1	Q16610	0.14380	0.27884	5,534.0724	3,723.9318	-1,953.3943	13,021.539094	-0.627900
CEP57L1	Q8IYX8	0.14475	0.27917	691.5349	466.4752	-246.3764	1,629.446098	-0.472069
TCP11L1	Q9NUJ3	0.14819	0.28331	258.2424	175.7200	-95.0662	611.551071	-0.358856
	P0DOX5	0.14884	0.28361	1,038.9070	708.0849	-384.7931	2,462.607009	-0.173171
CDH5	P33151	0.15451	0.29336	1,157.6914	800.2861	-451.3917	2,766.774480	-0.079819
PPIA	P62937	0.16440	0.30865	-138.2237	97.8910	-335.0468	58.599321	0.305037

PTGDS	P41222	0.16643	0.31067	-889.3801	632.9649	-2,162.0414	383.281244	0.188105
NQO1	P15559	0.16871	0.31453	606.2589	433.8350	-266.0249	1,478.542716	-0.092289
PRDX1	Q06830	0.17170	0.31834	757.6369	546.0540	-340.2783	1,855.552010	-1.574698
COMP	P49747	0.17333	0.31843	-11,212.6270	8,112.4958	-27,523.8931	5,098.639023	0.141918
GPX3	P22352	0.17483	0.32036	643.0822	466.9420	-295.7677	1,581.932055	-0.144766
ZNF555	Q8NEP9	0.17706	0.32226	2,252.6840	1,644.2784	-1,053.3592	5,558.727219	-0.194318
ISM1	B1AKI9	0.19399	0.34205	19,385.7715	14,716.2363	-10,203.2048	48,974.747783	-0.210134
DBH	P09172	0.19315	0.34209	160.7144	121.7691	-84.1188	405.547697	-0.336844
APOH	P02749	0.19236	0.34213	26,823.5476	20,286.8280	-13,965.8540	67,612.949072	-0.117094
HRG	P04196	0.19034	0.34220	-23,200.9835	17,465.7380	-58,318.2035	11,916.236391	0.161323
IGLV1-47	P01700	0.18950	0.34224	-1,922.9108	1,444.7791	-4,827.8339	982.012302	3.240864
IGFBP4	P22692	0.19221	0.34396	3,275.2338	2,476.2323	-1,703.5649	8,254.032573	-0.215111
PRAMEF19	Q5SWL8	0.19698	0.34552	-167.0978	127.7137	-423.8834	89.687703	0.241938
DES	P17661	0.20034	0.34715	-563.6480	434.1004	-1,436.4653	309.169365	0.118229
F13A1	P00488	0.19852	0.34721	-625.7430	479.9406	-1,590.7283	339.242325	0.284389
AZGP1	P25311	0.20168	0.34881	12,829.8990	9,911.0583	-7,097.6194	32,757.417326	-0.164202
GPLD1	P80108	0.20504	0.35038	-498.3326	387.8798	-1,278.2171	281.551904	0.108969
FAN1	Q9Y2M0	0.20429	0.35046	2,081.0936	1,617.0872	-1,170.2781	5,332.465346	-0.120223
CD33	P20138	0.20822	0.35192	244.2880	191.5011	-140.7509	629.326805	-2.604466
CFD	P00746	0.20759	0.35371	-1,212.1810	948.9158	-3,120.1040	695.742048	0.251988
ZGRF1	Q86YA3	0.21696	0.36530	-252.1436	201.5380	-657.3628	153.075632	0.155500
VCL	P18206	0.22013	0.36667	-458.1725	368.7863	-1,199.6671	283.322055	4.546279
ATP5F1B	P06576	0.21855	0.36683	-3,520.6703	2,823.9741	-9,198.6507	2,157.310087	0.295914

CFB	P00751	0.22367	0.37149	17,565.8421	14,249.1859	-11,084.0663	46,215.750601	-0.092367
PROC	P04070	0.22588	0.37296	690.8610	563.1444	-441.4168	1,823.138727	-0.171094
C9	P02748	0.23008	0.37770	-4,711.9479	3,876.2083	-12,505.5870	3,081.691214	0.097265
APOE	P02649	0.24156	0.39546	-2,250.1909	1,897.7206	-6,065.8139	1,565.432044	0.165825
C1QB	P02746	0.25183	0.40981	-4,099.8621	3,534.6594	-11,206.7712	3,007.047050	0.104025
MEPE	Q9NQ76	0.25509	0.41268	418.6763	363.4842	-312.1576	1,149.510234	-1.010974
YWHAZ	P63104	0.26554	0.42841	-106.7006	94.7156	-297.1390	83.737909	0.208170
MED4	Q9NPJ6	0.27176	0.43598	-428.5397	385.4467	-1,203.5324	346.452874	0.374706
SCIN	Q9Y6U3	0.28353	0.45305	-124.4519	114.7467	-355.1656	106.261894	0.107295
NRXN1	Q9ULB1	0.28834	0.45725	-277.1105	258.0980	-796.0513	241.830338	0.369234
CSTB	P04080	0.30533	0.48196	-273.0153	263.4910	-802.7995	256.768834	2.138867
HSP90B1	P14625	0.31133	0.48913	-529.1440	517.1285	-1,568.9005	510.612601	0.067437
CLEC3B	P05452	0.32127	0.50250	7,784.6292	7,767.4984	-7,832.9731	23,402.231524	-0.424006
TGFBI	Q15582	0.32440	0.50250	343.1502	344.6391	-349.7932	1,036.093614	-0.136412
HIBADH	P31937	0.32311	0.50328	-1,561.7039	1,564.2749	-4,706.8895	1,583.481639	0.060596
HK2	P52789	0.32709	0.50482	179.8335	181.6319	-185.3619	545.028871	-0.167487
A1BG	P04217	0.32878	0.50557	35,179.8113	35,656.9679	-36,513.3277	106,872.950267	-0.148484
PTCD3	Q96EY7	0.33639	0.51164	-1,880.9238	1,936.9918	-5,775.5068	2,013.659160	0.119218
LRG1	P02750	0.33610	0.51397	-2,178.7613	2,242.3571	-6,687.3225	2,329.799856	0.114883
PVALB	P20472	0.34216	0.51842	469.2375	489.0907	-514.1452	1,452.620247	-2.924357
PSMC3IP	Q9P2W1	0.35284	0.53029	-1,165.8422	1,242.6515	-3,664.3605	1,332.676088	0.083842
B2M	P61769	0.35787	0.53066	-444.6420	478.9541	-1,407.6438	518.359821	0.095190
LYZ	P61626	0.35478	0.53092	614.9478	658.1341	-708.3194	1,938.214975	-0.154706

PRDX2	P32119	0.35271	0.53268	434.5653	463.0610	-496.4813	1,365.611873	-0.141237
ARHGAP8	P85298	0.35751	0.53302	-4,060.7080	4,370.7632	-12,848.7165	4,727.300427	0.123575
RAB11FIP4	Q86YS3	0.37212	0.54658	126.9012	140.8557	-156.3082	410.110636	-0.144665
HBA1	P69905	0.37123	0.54751	8,261.2287	9,152.4075	-10,140.9200	26,663.377396	-2.596363
TTF1	Q15361	0.38315	0.56028	357.2015	405.8284	-458.7711	1,173.174070	-0.153892
PGLYRP2	Q96PD5	0.39334	0.57241	5,567.7644	6,464.1143	-7,429.2084	18,564.737245	-0.117094
PZP	P20742	0.39601	0.57429	-525.2878	613.3339	-1,758.4782	707.902533	0.075767
TNFSF11	O14788	0.39997	0.57759	177.8614	209.4367	-243.2393	598.962193	-0.089042
LYVE1	Q9Y5Y7	0.41049	0.58948	-71.0748	85.6028	-243.1908	101.041115	0.196379
DNAH8	Q96JB1	0.42049	0.59872	443.9402	546.3509	-654.5720	1,542.452340	-0.061103
TUBA1B	P68363	0.42163	0.59903	64.2166	79.2271	-95.0801	223.513236	-0.329717
SACS	Q9NZJ4	0.42383	0.59932	2,659.6962	3,297.0739	-3,969.5151	9,288.907548	-0.084641
CA2	P00918	0.42566	0.59962	295.6660	367.9849	-444.2172	1,035.549189	-0.089260
APOF	Q13790	0.41875	0.59985	-62.4510	76.5693	-216.4039	91.501985	0.120613
GSN	P06396	0.43092	0.60412	12,559.2104	15,811.4713	-19,231.8835	44,350.304211	-0.095893
GPATCH2L	Q9NWQ4	0.43624	0.60606	84.7912	107.9985	-132.3544	301.936804	-0.151635
RALGAPB	Q86X10	0.43810	0.60632	1,259.7913	1,611.1442	-1,979.6313	4,499.213870	-0.191935
ICAM1	P05362	0.43546	0.60719	-70.1491	89.1945	-249.4866	109.188388	0.097796
LPA	P08519	0.44667	0.61623	443.9402	578.5852	-719.3833	1,607.263663	-0.109568
TMEM35B	Q8NCS4	0.45956	0.63156	-105.0053	140.8375	-388.1781	178.167568	0.174039
BASP1	P80723	0.46536	0.63582	24,589.0423	33,412.3045	-42,590.8984	91,768.983038	-0.609162
USP31	Q70CQ4	0.47151	0.64276	923.2500	1,272.1105	-1,634.4996	3,480.999712	-0.053899
CST3	P01034	0.47399	0.64287	116.6660	161.6574	-208.3679	441.699964	-0.136658

AHSG	P02765	0.49814	0.67000	22,393.7051	32,806.7253	-43,568.6371	88,356.047316	-0.087583
NCAM1	P13591	0.49630	0.67000	115.8260	168.9561	-223.8831	455.535046	-0.257431
ANPEP	P15144	0.49975	0.67000	-422.4683	621.2445	-1,671.5641	826.627429	0.111544
KNTC1	P50748	0.50437	0.67267	141.2132	209.9233	-280.8659	563.292313	-0.078428
C5	P01031	0.51629	0.68595	-8,527.6271	13,041.0271	-34,748.3695	17,693.115332	0.100546
TRBV24-1	A0A075B6N3	0.52418	0.69110	369.1495	575.3554	-787.6801	1,525.979159	-1.687526
PROCR	Q9UNN8	0.52324	0.69251	-166.8409	259.4432	-688.4864	354.804628	0.196741
DYTN	A2CJ06	0.53288	0.70022	218.9403	348.5491	-481.8647	919.745310	-0.200111
ALDH1A3	P47895	0.53619	0.70141	509.4101	817.5840	-1,134.4528	2,153.272967	-0.129190
SEC14L1	Q92503	0.54245	0.70650	-181.1171	295.2274	-774.7115	412.477315	0.303583
OPRL1	P41146	0.54713	0.71025	-2,484.3221	4,097.0291	-10,721.9512	5,753.307050	0.075678
CABIN1	Q9Y6J0	0.54970	0.71139	-352.6942	585.4219	-1,529.7639	824.375432	0.306712
F12	P00748	0.57443	0.73393	2,320.7332	4,104.5406	-5,931.9988	10,573.465198	-0.085389
LIFR	P42702	0.57153	0.73418	546.7233	959.6579	-1,382.7983	2,476.244769	-0.055708
CFHR1	Q03591	0.56986	0.73442	1,626.2239	2,842.0693	-4,088.1394	7,340.587171	-0.078299
TELO2	Q9Y4R8	0.60676	0.77317	-316.6251	611.1057	-1,545.3354	912.085244	0.100322
POTEE	Q6S8J3	0.61041	0.77405	-60.5845	118.1347	-298.1103	176.941206	0.042004
SPTBN1	Q01082	0.63410	0.79846	-1,533.9836	3,202.3327	-7,972.7051	4,904.737837	0.740523
MYH9	P35579	0.63916	0.79875	954.8491	2,023.5354	-3,113.7417	5,023.439753	-0.136999
CRISP3	P54108	0.63203	0.79894	-86.2885	179.0378	-446.2680	273.691123	0.060883
TUT7	Q5VYS8	0.64538	0.80028	583.7547	1,260.5108	-1,950.6721	3,118.181450	-0.051828
TUBB	P07437	0.63751	0.80049	-58.3063	122.9593	-305.5325	188.919862	0.082816
PRAG1	Q86YV5	0.64511	0.80325	1,912.6275	4,126.5664	-6,384.3904	10,209.645422	-1.094915

GPX1	P07203	0.66752	0.81080	-47.8837	110.7880	-270.6379	174.870466	0.088364
CPN2	P22792	0.67301	0.81099	1,330.6494	3,133.7108	-4,970.0985	7,631.397262	-0.061796
PFN1	P07737	0.66624	0.81131	-409.9463	944.5959	-2,309.1836	1,489.291048	0.036529
ARRDC3	Q96B67	0.67181	0.81271	214.2572	502.6287	-796.3455	1,224.859858	-0.075249
B3GNT6	Q6ZMB0	0.66034	0.81287	-142.1130	321.3851	-788.3010	504.074953	0.114947
TNXB	P22105	0.66486	0.81305	399.9671	917.5705	-1,444.9320	2,244.866244	-0.045200
DPY19L2P2	Q6ZN68	0.66395	0.81480	18.6943	42.7618	-67.2841	104.672755	-0.180748
PLGLA	Q15195	0.65958	0.81587	-197.1055	444.6826	-1,091.1998	696.988825	0.085420
AFM	P43652	0.68712	0.81612	-1,746.7465	4,310.6170	-10,413.8229	6,920.329882	0.031959
PEBP4	Q96S96	0.68085	0.81769	71.1841	172.0186	-274.6825	417.050701	-0.124632
FN1	P02751	0.68631	0.81783	-6,266.2452	15,421.8901	-37,274.0334	24,741.543016	0.051455
BTD	P43251	0.69080	0.81797	564.0715	1,409.5370	-2,269.9926	3,398.135569	-0.096418
DNHD1	Q96M86	0.68467	0.81955	-255.7297	625.9189	-1,514.2241	1,002.764639	0.109312
FBLN1	P23142	0.69655	0.82217	625.4197	1,594.1260	-2,579.7853	3,830.624802	-0.033675
PIKFYVE	Q9Y2I7	0.70749	0.82813	-128.6799	340.9016	-814.1084	556.748623	0.032105
GLI1	P08151	0.70530	0.82868	807.9023	2,123.6195	-3,461.9210	5,077.725490	-0.092374
ZMYND8	Q9ULU4	0.71234	0.83108	-215.0227	579.7138	-1,380.6154	950.569964	0.045647
MASP1	P48740	0.71684	0.83112	-219.8136	602.5084	-1,431.2379	991.610648	0.053017
RASAL1	O95294	0.71657	0.83401	194.5951	532.8565	-876.7846	1,265.974776	-0.155544
IGHV3-49	A0A0A0MS15	0.72353	0.83634	-1,515.8037	4,259.9177	-10,080.9424	7,049.334961	0.032633
PPBP	P02775	0.73284	0.84383	752.4542	2,191.5423	-3,653.9369	5,158.845266	-0.437795
APOD	P05090	0.73578	0.84438	-35.8034	105.4850	-247.8953	176.288474	0.081998
C8A	P07357	0.74090	0.84722	650.6426	1,956.3435	-3,282.8496	4,584.134808	-0.025555

IQCE	Q6IPM2	0.75529	0.86029	179.5547	572.8128	-972.1626	1,331.271974	-0.065266
CFHR2	P36980	0.76810	0.87214	1,259.6086	4,247.7074	-7,280.9795	9,800.196745	-0.052436
VWF	P04275	0.77068	0.87258	-695.5061	2,372.6146	-5,465.9675	4,074.955209	0.038619
PIBF1	Q8WXW3	0.78690	0.87590	-35.7076	131.3469	-299.7982	228.383114	0.020408
LCP1	P13796	0.78631	0.87770	-249.2931	914.4245	-2,087.8668	1,589.280586	0.067803
CFHR5	Q9BXR6	0.78490	0.87952	161.4642	588.2647	-1,021.3214	1,344.249723	-0.044625
SERPINF1	P36955	0.78109	0.88093	1,081.7843	3,866.6696	-6,732.1961	8,895.764768	-0.021427
IL1RAP	Q9NPH3	0.78447	0.88134	1,166.5397	4,241.4056	-7,361.3778	9,694.457279	-0.278456
CD5L	O43866	0.79482	0.88187	-292.4574	1,118.3379	-2,541.0265	1,956.111715	0.025522
EEF1A1	P68104	0.81032	0.88388	36.7447	152.2518	-269.3780	342.867377	-0.020422
KIAA2026	Q5HYC2	0.80950	0.88567	152.3405	628.4572	-1,111.2573	1,415.938290	-0.143534
LUM	P51884	0.81729	0.88574	-1,928.4037	8,301.1157	-18,618.9154	14,762.107978	0.086078
VNN1	O95497	0.80731	0.88638	540.2664	2,202.9081	-3,888.9773	4,969.510072	-0.427880
ZNHIT2	Q9UHR6	0.81543	0.88644	204.7102	872.1546	-1,548.8742	1,958.294498	-0.025624
APOM	O95445	0.82338	0.88651	-584.6036	2,604.8807	-5,822.0672	4,652.859960	0.129147
GPR55	Q9Y2T6	0.80567	0.88819	43.0802	174.1482	-307.0682	393.228596	-0.054081
CDK5RAP2	Q96SN8	0.82236	0.88829	-101.5872	450.0202	-1,006.4134	803.238971	0.024026
RNF213	Q63HN8	0.80434	0.88891	3,559.0176	14,286.7222	-25,166.3626	32,284.397754	-0.115447
GID8	Q9NWU2	0.83885	0.89511	58.0960	284.1354	-513.1964	629.388393	-0.267708
CEP350	Q5VT06	0.83761	0.89690	-1,237.2260	6,003.9569	-13,308.9904	10,834.538414	0.018324
MST1	P26927	0.83585	0.89763	177.0620	849.8894	-1,531.7553	1,885.879202	-0.024676
SERPINA6	P08185	0.85079	0.90503	51.7888	273.8246	-498.7725	602.349996	-0.013305
CFAP43	Q8NDM7	0.85995	0.90883	-457.8046	2,580.7367	-5,646.7235	4,731.114198	0.013443

PKN2	Q16513	0.85787	0.90959	-185.4445	1,029.9194	-2,256.2363	1,885.347346	0.027257
UBB	P0CG47	0.88651	0.92281	21.3563	148.8460	-277.9187	320.631193	-0.103521
	P0DOX2	0.88464	0.92360	-19.0464	130.5824	-281.5999	243.507072	0.055392
HSPA8	P11142	0.88106	0.92519	44.8376	298.0838	-554.5000	644.175175	-0.072517
LDHB	P07195	0.88437	0.92544	-7.2280	49.4353	-106.6244	92.168398	0.016377
PCSK1	P29120	0.88088	0.92810	23.3482	154.9806	-288.2612	334.957617	-0.009567
PI16	Q6UXB8	0.89834	0.93136	1,613.7746	12,565.1692	-23,650.1913	26,877.740514	-0.176786
C8G	P07360	0.91387	0.94503	312.2703	2,872.0843	-5,462.4423	6,086.982873	-0.009536
APOA4	P06727	0.92670	0.95259	-902.9705	9,763.8098	-20,534.4259	18,728.484929	0.016312
IGHG4	P01861	0.92475	0.95346	297.9575	3,138.2213	-6,011.8593	6,607.774356	-0.020120
SERPINA5	P05154	0.93675	0.95700	-231.5745	2,902.7386	-6,067.9217	5,604.772657	0.010752
MYH14	Q7Z406	0.93601	0.95890	23.6657	293.2447	-565.9422	613.273735	-0.009457
PLG	P00747	0.95873	0.97649	-641.6264	12,333.9741	-25,440.7433	24,157.490586	0.004436
SERPING1	P05155	0.96261	0.97759	-183.7452	3,899.6188	-8,024.4544	7,656.963915	0.004634
VCAM1	P19320	0.97027	0.98172	-18.4898	493.5431	-1,010.8248	973.845220	0.005211
F5	P12259	0.98477	0.98500	385.2093	20,079.9810	-39,988.2985	40,758.717087	-0.020724
IGLC6	P0CF74	0.98330	0.98594	5.7270	272.0992	-541.3652	552.819133	-0.004192
EMD	P50402	0.97885	0.98785	10.2138	383.3272	-760.5173	780.944844	-0.003209
CRTAC1	Q9NQ79	0.98207	0.98790	-11.5454	511.0558	-1,039.0919	1,016.001068	0.004962

Supplementary table 2: 69 biological processes and 24 molecular functions related to LLD profiling					
Biological Process	corrected p-val	p-val	cluster frequency	total frequency	Gene names
acute inflammatory response	7.42E-05	2.02E-07	5/22 22.7%	89/14306 0.6%	O75636 P18428 P06681 P09871 P05156
complement activation	7.42E-05	3.70E-07	4/22 18.1%	40/14306 0.2%	O75636 P06681 P09871 P05156
activation of plasma proteins involved in acute inflammatory response	7.42E-05	4.09E-07	4/22 18.1%	41/14306 0.2%	O75636 P06681 P09871 P05156
immune effector process	1.75E-04	1.28E-06	5/22 22.7%	129/14306 0.9%	O75636 P18428 P06681 P09871 P05156
positive regulation of immune system process	1.84E-04	2.22E-06	6/22 27.2%	265/14306 1.8%	O75636 P18428 Q5JRA6 P06681 P09871 P05156
positive regulation of immune response	1.84E-04	2.37E-06	5/22 22.7%	146/14306 1.0%	O75636 P18428 P06681 P09871 P05156
positive regulation of response to stimulus	1.84E-04	2.37E-06	6/22 27.2%	268/14306 1.8%	O75636 P18428 P06681 P09871 P05156 P01344
positive regulation of biological process	2.33E-04	3.43E-06	13/22 59.0%	2208/14306 15.4%	P27169 O75636 Q5JRA6 Q13617 P18428 P17936 P06681 P00742 P09871 P05156 P08238 P01344 O14556
innate immune response	2.69E-04	4.45E-06	5/22 22.7%	166/14306 1.1%	O75636 P18428 P06681 P09871 P05156
humoral immune response	2.87E-04	5.27E-06	4/22 18.1%	77/14306 0.5%	O75636 P06681 P09871 P05156
protein maturation by peptide bond cleavage	3.19E-04	6.46E-06	4/22 18.1%	81/14306 0.5%	O75636 P06681 P09871 P05156
regulation of response to stimulus	4.07E-04	8.97E-06	7/22 31.8%	524/14306 3.6%	O75636 P18428 Q16512 P06681 P09871 P05156 P01344
response to wounding	4.37E-04	1.11E-05	7/22 31.8%	541/14306 3.7%	O75636 P18428 Q5JRA6 P06681 P00742 P09871 P05156
complement activation, classical pathway	4.37E-04	1.12E-05	3/22 13.6%	29/14306 0.2%	P06681 P09871 P05156
activation of immune response	4.60E-04	1.27E-05	4/22 18.1%	96/14306 0.6%	O75636 P06681 P09871 P05156
humoral immune response mediated by circulating immunoglobulin	4.69E-04	1.38E-05	3/22 13.6%	31/14306 0.2%	P06681 P09871 P05156
protein processing	5.79E-04	1.81E-05	4/22 18.1%	105/14306 0.7%	O75636 P06681 P09871 P05156
regulation of immune response	7.26E-04	2.40E-05	5/22 22.7%	235/14306 1.6%	O75636 P18428 P06681 P09871 P05156
protein maturation	7.42E-04	2.59E-05	4/22 18.1%	115/14306 0.8%	O75636 P06681 P09871 P05156
regulation of immune system process	8.87E-04	3.26E-05	6/22 27.2%	424/14306 2.9%	O75636 P18428 Q5JRA6 P06681 P09871 P05156

immunoglobulin mediated immune response	1.53E-03	5.90E-05	3/22 13.6%	50/14306 0.3%	P06681 P09871 P05156
B cell mediated immunity	1.55E-03	6.27E-05	3/22 13.6%	51/14306 0.3%	P06681 P09871 P05156
inflammatory response	2.29E-03	9.70E-05	5/22 22.7%	315/14306 2.2%	O75636 P18428 P06681 P09871 P05156
lymphocyte mediated immunity	2.68E-03	1.18E-04	3/22 13.6%	63/14306 0.4%	P06681 P09871 P05156
primary metabolic process	2.92E-03	1.38E-04	17/22 77.2%	5288/14306 36.9%	P27169 O75636 Q92945 Q16512 Q13617 O00750 Q13627 P19827 P17936 P06681 P00742 P09871 P05156 P08238 Q9ULZ9 P01344 O14556
metabolic process	2.92E-03	1.40E-04	18/22 81.8%	5959/14306 41.6%	P27169 O75636 Q92945 Q16512 Q13617 O00750 Q13627 P00915 P19827 P17936 P06681 P00742 P09871 P05156 P08238 Q9ULZ9 P01344 O14556
adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	3.26E-03	1.62E-04	3/22 13.6%	70/14306 0.4%	P06681 P09871 P05156
adaptive immune response	3.28E-03	1.69E-04	3/22 13.6%	71/14306 0.4%	P06681 P09871 P05156
immune response	4.86E-03	2.64E-04	6/22 27.2%	619/14306 4.3%	O75636 P18428 P06681 P15812 P09871 P05156
leukocyte mediated immunity	4.86E-03	2.68E-04	3/22 13.6%	83/14306 0.5%	P06681 P09871 P05156
immune system process	6.80E-03	3.87E-04	7/22 31.8%	948/14306 6.6%	O75636 P18428 P19827 P06681 P15812 P09871 P05156
positive regulation of protein kinase B signaling cascade	9.52E-03	5.60E-04	2/22 9.0%	23/14306 0.1%	P00742 P01344
proteolysis	1.05E-02	6.38E-04	6/22 27.2%	730/14306 5.1%	P06681 Q13617 P00742 P09871 P05156 Q9ULZ9
protein metabolic process	1.13E-02	7.09E-04	11/22 50.0%	2611/14306 18.2%	O75636 Q16512 P17936 P06681 Q13617 P00742 P09871 Q13627 P05156 P08238 Q9ULZ9
response to stress	1.15E-02	7.39E-04	9/22 40.9%	1773/14306 12.3%	O75636 P18428 Q16512 Q5JRA6 P06681 P00742 P09871 P05156 P08238
regulation of protein kinase B signaling cascade	1.65E-02	1.09E-03	2/22 9.0%	32/14306 0.2%	P00742 P01344
leukocyte chemotaxis involved in inflammatory response	2.15E-02	1.54E-03	1/22 4.5%	1/14306 0.0%	P18428
histone H3-T11 phosphorylation	2.15E-02	1.54E-03	1/22 4.5%	1/14306 0.0%	Q16512
positive regulation of respiratory burst involved in inflammatory response	2.15E-02	1.54E-03	1/22 4.5%	1/14306 0.0%	P18428
defense response	2.91E-02	2.14E-03	5/22 22.7%	620/14306 4.3%	O75636 P18428 P06681 P09871 P05156

macromolecule metabolic process	2.99E-02	2.25E-03	13/22 59.0%	4017/14306 28.0%	O75636 Q92945 Q16512 Q13617 Q13627 P19827 P17936 P06681 P00742 P09871 P05156 P08238 Q9ULZ9
regulation of biological process	3.34E-02	2.70E-03	17/22 77.2%	6554/14306 45.8%	P27169 O75636 Q92945 Q16512 Q5JRA6 Q13617 O00750 P18428 P41594 P17936 P06681 P00742 P09871 P05156 P08238 P01344 O14556
activation of phospholipase C activity by metabotropic glutamate receptor signaling pathway	3.34E-02	3.07E-03	1/22 4.5%	2/14306 0.0%	P41594
regulation of toll-like receptor 4 signaling pathway	3.34E-02	3.07E-03	1/22 4.5%	2/14306 0.0%	P18428
positive regulation of toll-like receptor 4 signaling pathway	3.34E-02	3.07E-03	1/22 4.5%	2/14306 0.0%	P18428
polysaccharide transport	3.34E-02	3.07E-03	1/22 4.5%	2/14306 0.0%	P18428
leukocyte migration involved in inflammatory response	3.34E-02	3.07E-03	1/22 4.5%	2/14306 0.0%	P18428
lipopolysaccharide transport	3.34E-02	3.07E-03	1/22 4.5%	2/14306 0.0%	P18428
regulation of respiratory burst involved in inflammatory response	3.34E-02	3.07E-03	1/22 4.5%	2/14306 0.0%	P18428
regulation of type I interferon-mediated signaling pathway	3.34E-02	3.07E-03	1/22 4.5%	2/14306 0.0%	P08238
response to stimulus	3.50E-02	3.29E-03	12/22 54.5%	3633/14306 25.3%	P27169 O75636 P18428 Q16512 Q5JRA6 P06681 P15812 P00742 P09871 P05156 P08238 P01344
regulation of cell migration	4.40E-02	4.23E-03	3/22 13.6%	216/14306 1.5%	P17936 Q5JRA6 P00742
polysaccharide localization	4.40E-02	4.61E-03	1/22 4.5%	3/14306 0.0%	P18428
positive regulation of insulin receptor signaling pathway	4.40E-02	4.61E-03	1/22 4.5%	3/14306 0.0%	P01344
cellular response to lipoteichoic acid	4.40E-02	4.61E-03	1/22 4.5%	3/14306 0.0%	P18428
histone-threonine phosphorylation	4.40E-02	4.61E-03	1/22 4.5%	3/14306 0.0%	Q16512
positive regulation of respiratory burst	4.40E-02	4.61E-03	1/22 4.5%	3/14306 0.0%	P18428
negative regulation of cell migration	4.41E-02	4.70E-03	2/22 9.0%	67/14306 0.4%	P17936 Q5JRA6
negative regulation of cellular component movement	4.81E-02	5.41E-03	2/22 9.0%	72/14306 0.5%	P17936 Q5JRA6
negative regulation of locomotion	4.81E-02	5.41E-03	2/22 9.0%	72/14306 0.5%	P17936 Q5JRA6
regulation of signal transduction	4.81E-02	5.55E-03	5/22 22.7%	773/14306 5.4%	Q16512 P17936 P00742 P08238 P01344

regulation of cellular component movement	4.81E-02	5.61E-03	3/22 13.6%	239/14306 1.6%	P17936 Q5JRA6 P00742
regulation of signaling process	4.81E-02	5.70E-03	5/22 22.7%	778/14306 5.4%	Q16512 P17936 P00742 P08238 P01344
regulation of locomotion	4.81E-02	5.74E-03	3/22 13.6%	241/14306 1.6%	P17936 Q5JRA6 P00742
biological regulation	4.81E-02	5.75E-03	17/22 77.2%	6943/14306 48.5%	P27169 O75636 Q92945 Q16512 Q5JRA6 Q13617 O00750 P18428 P41594 P17936 P06681 P00742 P09871 P05156 P08238 P01344 O14556
positive regulation of macrophage activation	4.91E-02	6.14E-03	1/22 4.5%	4/14306 0.0%	P18428
organophosphate catabolic process	4.91E-02	6.14E-03	1/22 4.5%	4/14306 0.0%	P27169
regulation of interferon-gamma-mediated signaling pathway	4.91E-02	6.14E-03	1/22 4.5%	4/14306 0.0%	P08238
Biological Process	corrected p-val	p-val	cluster frequency	total frequency	Gene names
serine-type endopeptidase activity	5.11E-05	4.08E-03	4/21 19.0%	155/15444 1.0%	P06681 P00742 P09871 P05156
serine-type peptidase activity	8.57E-05	4.08E-03	4/21 19.0%	177/15444 1.1%	P06681 P00742 P09871 P05156
serine hydrolase activity	9.15E-05	4.08E-03	4/21 19.0%	180/15444 1.1%	P06681 P00742 P09871 P05156
endopeptidase activity	1.18E-04	4.08E-03	5/21 23.8%	373/15444 2.4%	P06681 P00742 P09871 P05156 Q9ULZ9
peptidase activity, acting on L-amino acid peptides	7.18E-04	1.93E-02	5/21 23.8%	551/15444 3.5%	P06681 P00742 P09871 P05156 Q9ULZ9
peptidase activity	8.37E-04	1.93E-02	5/21 23.8%	570/15444 3.6%	P06681 P00742 P09871 P05156 Q9ULZ9
calcium ion binding	1.28E-03	2.35E-02	5/21 23.8%	626/15444 4.0%	P27169 P19827 P00742 P09871 Q9ULZ9
histone kinase activity (H3-T11 specific)	1.36E-03	2.35E-02	1/21 4.7%	1/15444 0.0%	Q16512
phospholipid binding	1.98E-03	2.68E-02	3/21 14.2%	187/15444 1.2%	P27169 O00750 P00742
lipid binding	2.07E-03	2.68E-02	4/21 19.0%	411/15444 2.6%	P27169 P18428 O00750 P00742
glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity	2.72E-03	2.68E-02	1/21 4.7%	2/15444 0.0%	O14556
protein tyrosine phosphatase activator activity	2.72E-03	2.68E-02	1/21 4.7%	2/15444 0.0%	P17936
lipoteichoic acid binding	2.72E-03	2.68E-02	1/21 4.7%	2/15444 0.0%	P18428

glyceraldehyde-3-phosphate dehydrogenase activity	2.72E-03	2.68E-02	1/21 4.7%	2/15444 0.0%	O14556
histone threonine kinase activity	4.07E-03	3.12E-02	1/21 4.7%	3/15444 0.0%	Q16512
phosphatidylinositol-4-phosphate 3-kinase activity	4.07E-03	3.12E-02	1/21 4.7%	3/15444 0.0%	O00750
TPR domain binding	4.07E-03	3.12E-02	1/21 4.7%	3/15444 0.0%	P08238
aryldialkylphosphatase activity	4.07E-03	3.12E-02	1/21 4.7%	3/15444 0.0%	P27169
Gram-negative bacterial cell surface binding	5.43E-03	3.57E-02	1/21 4.7%	4/15444 0.0%	P18428
Gram-positive bacterial cell surface binding	5.43E-03	3.57E-02	1/21 4.7%	4/15444 0.0%	P18428
arylesterase activity	5.43E-03	3.57E-02	1/21 4.7%	4/15444 0.0%	P27169
phosphatase activator activity	6.78E-03	4.07E-02	1/21 4.7%	5/15444 0.0%	P17936
nitric-oxide synthase regulator activity	6.78E-03	4.07E-02	1/21 4.7%	5/15444 0.0%	P08238
insulin-like growth factor I binding	8.13E-03	4.68E-02	1/21 4.7%	6/15444 0.0%	P17936

Supplementary table 3: Cognitive impairment related to LLD patients – Independent sample t-test						
					95% Confidence Interval of the Difference	
Accession	Sig. (2-tailed)	Benjamini-Hochberg Adjusted P value	Mean Difference	Std. Error Difference	Lower	Upper
P0DPA3	0.0017880	0.599000	3,593.5745	971.92662	1,542.9886	5,644.1604
P04114	0.0027114	0.454000	102,158.9491	29,142.29386	40,674.0836	163,643.8147
Q5VT06	0.0047701	0.533000	27,296.8493	8,413.78976	9,545.3046	45,048.3940
Q9H254	0.0092080	0.771000	293.4551	99.91257	82.6581	504.2522
Q96KN2	0.0101156	0.678000	3,711.1670	1,282.89624	1,004.4925	6,417.8414
P04275	0.0143934	0.804000	11,750.7028	4,311.70236	2,653.8060	20,847.5996
Q562R1	0.0236992	1.000000	786.8371	316.73111	118.5929	1,455.0813
Q9Y6U3	0.0262243	1.000000	499.3595	205.11405	66.6067	932.1123
P29120	0.0307338	1.000000	529.2845	224.64907	55.3164	1,003.2526
P07225	0.0328755	1.000000	17,619.2398	7,586.37896	1,613.3793	33,625.1003
P04196	0.0338164	1.000000	75,834.6316	32,851.91702	6,523.1453	145,146.1179
Q96QS6	0.0385188	1.000000	1,976.8228	881.35740	117.3213	3,836.3244
P43652	0.0392210	1.000000	15,378.1782	6,884.31416	853.5449	29,902.8114
P50402	**0.0410774**	**0.983000**	**1,424.9440**	**644.66313**	**64.8237**	**2,785.0643**
P0DOX7	0.0523743	1.000000	-4,526.8113	2,170.25963	-9,105.6589	52.0363
P01031	0.0542744	1.000000	-41,660.0790	20,151.40830	-84,175.8341	855.6761
Q96EY7	0.0557121	1.000000	7,774.8426	3,785.67432	-212.2321	15,761.9173
Q63HN8	0.0569665	1.000000	42,154.1514	20,642.09280	-1,396.8576	85,705.1603
P49747	0.0769032	1.000000	21,870.5243	11,613.67275	-2,632.1834	46,373.2320
P04070	0.0776198	1.000000	-1,033.6619	550.35421	-2,194.8078	127.4840

Q9NZP8	0.0787066	1.000000	1,066.5100	570.11932	-136.3366	2,269.3566
P00488	0.0828700	1.000000	1,350.6049	732.91687	-195.7146	2,896.9243
P80108	0.0847971	1.000000	1,129.2138	616.96065	-172.4594	2,430.8870
Q9UK55	0.0848779	1.000000	-2,189.1391	1,196.40369	-4,713.3303	335.0520
Q9Y2T6	0.0904805	1.000000	523.1977	291.50184	-91.8174	1,138.2129
P04217	0.0928285	1.000000	-96,317.6427	54,088.02563	-210,433.4017	17,798.1164
Q13790	0.1022697	1.000000	265.1453	153.51893	-58.7513	589.0419
P01042	0.1081974	1.000000	31,579.1412	18,624.18508	-7,714.4546	70,872.7370
P10909	0.1140266	1.000000	10,422.5976	6,255.97313	-2,776.3519	23,621.5472
Q9Y2M0	0.1195683	1.000000	-4,051.2972	2,471.68054	-9,266.0873	1,163.4929
Q9P2W1	0.1205447	1.000000	3,182.6219	1,947.21154	-925.6353	7,290.8792
O60308	0.1227216	1.000000	-9,200.9405	5,664.79854	-21,152.6207	2,750.7397
Q15361	0.1336107	1.000000	1,104.4714	701.11411	-374.7500	2,583.6929
P0C0L5	0.1409740	1.000000	24,197.4573	15,670.88809	-8,865.2265	57,260.1411
Q9NVE5	0.1412882	1.000000	947.6990	614.27147	-348.3005	2,243.6985
O75019	0.1470682	1.000000	416.7842	274.32798	-161.9973	995.5656
P12814	0.1577635	1.000000	528.4397	357.59861	-226.0274	1,282.9068
P04003	0.1605532	1.000000	-36,602.3643	24,945.50674	-89,232.7830	16,028.0544
P02748	0.1619949	1.000000	-10,292.2086	7,040.05501	-25,145.4263	4,561.0091
P10643	0.1808757	1.000000	-14,692.9036	10,529.99186	-36,909.2445	7,523.4373
P47895	0.1913281	1.000000	1,365.0010	1,003.04489	-751.2387	3,481.2407
Q15063	0.1969924	1.000000	1,316.9863	980.77639	-752.2710	3,386.2436
Q13474	0.2073326	1.000000	73.2555	55.88284	-44.6470	191.1579

P85298	0.2137179	1.000000	7,838.5952	6,068.02576	-4,963.8201	20,641.0104
P41146	0.2193549	1.000000	8,041.9533	6,305.91153	-5,262.3571	21,346.2636
O00750	0.2198934	1.000000	-148.7772	116.80281	-395.2096	97.6552
P29622	0.2227684	1.000000	1,397.5724	1,104.37842	-932.4624	3,727.6072
Q9C056	0.2240108	1.000000	-125.9228	99.78527	-336.4514	84.6057
P01834	0.2309781	1.000000	-14,201.1827	11,431.03788	-38,318.5645	9,916.1990
P02100	0.2327823	1.000000	2,516.6873	2,033.95945	-1,774.5920	6,807.9667
P02751	0.2378899	1.000000	-47,886.0641	39,143.82934	-130,472.3251	34,700.1968
P08185	0.2381167	1.000000	512.6298	419.25358	-371.9180	1,397.1775
P58397	0.2439838	1.000000	-3,309.8797	2,742.38540	-9,095.8071	2,476.0477
P52789	0.2444791	1.000000	-371.9950	308.55192	-1,022.9826	278.9927
O14788	0.2463278	1.000000	484.4062	403.43336	-366.7637	1,335.5762
Q9Y2Q0	0.2558913	1.000000	773.4113	657.79181	-614.4082	2,161.2307
Q8WXW3	0.2690846	1.000000	259.8983	227.48308	-220.0491	739.8456
Q9NPY3	0.2716963	1.000000	-263.0043	231.50687	-751.4411	225.4325
Q9UHF7	0.2720436	1.000000	639.4317	563.27622	-548.9772	1,827.8407
P03951	0.2735402	1.000000	1,066.7165	942.71673	-922.2419	3,055.6750
P03952	0.2751624	1.000000	-3,434.4243	3,045.84413	-9,860.5937	2,991.7451
P54108	0.2783778	1.000000	428.2850	382.47118	-378.6586	1,235.2287
Q13561	0.2800206	1.000000	1,117.9441	1,001.89645	-995.8726	3,231.7608
P00740	0.2830276	1.000000	2,230.4125	2,011.86103	-2,014.2432	6,475.0683
Q9BYX7	0.2847463	1.000000	967.2926	875.73989	-880.3570	2,814.9423
P15559	0.2876129	1.000000	670.5505	610.83227	-618.1929	1,959.2940

A6NKP2	0.2977010	1.000000	-1,561.6137	1,453.61124	-4,628.4653	1,505.2379
P68104	0.2997130	1.000000	-320.9783	300.06697	-954.0643	312.1076
P13796	0.3022250	1.000000	-2,260.2176	2,124.33625	-6,742.1753	2,221.7401
P05154	0.3030932	1.000000	-5,552.5011	5,228.37460	-16,583.4073	5,478.4050
P49908	0.3076944	1.000000	257.4731	244.83521	-259.0840	774.0303
Q15195	0.3089661	1.000000	-1,197.9761	1,142.26760	-3,607.9501	1,211.9979
P00736	0.3126876	1.000000	-5,962.8728	5,730.86294	-18,053.9367	6,128.1911
P08519	0.3147149	1.000000	-1,531.4328	1,478.21637	-4,650.1967	1,587.3311
P08670	0.3167154	1.000000	168.8256	163.65477	-176.4558	514.1070
Q86YS3	0.3178265	1.000000	-364.3376	354.01510	-1,111.2441	382.5690
Q08380	0.3308648	1.000000	810.2485	809.44227	-897.5254	2,518.0224
P02741	0.3335800	1.000000	-2,397.5298	2,409.01966	-7,480.1170	2,685.0574
P02753	0.3355125	1.000000	24,948.2497	25,171.08824	-28,158.1044	78,054.6038
Q6S8J3	0.3368493	1.000000	178.0007	180.10214	-201.9816	557.9830
Q8IYW2	0.3392494	1.000000	-8,645.3193	8,792.17041	-27,195.1774	9,904.5388
P01861	0.3404228	1.000000	-4,345.4602	4,430.32259	-13,692.6238	5,001.7034
P0CG47	0.3413599	1.000000	-33.5862	34.31050	-105.9751	38.8026
P14373	0.3420582	1.000000	-4,715.6737	4,824.52928	-14,894.5407	5,463.1934
P01034	0.3440336	1.000000	-192.7965	198.07809	-610.7047	225.1118
O76027	0.3442209	1.000000	-301.1842	309.55850	-954.2956	351.9271
A0A0A0MS15	0.3472304	1.000000	7,761.6954	8,028.79015	-9,177.5711	24,700.9620
Q8TE12	0.3537676	1.000000	-3,546.6818	3,720.18978	-11,395.5962	4,302.2325
P20851	0.3554923	1.000000	-3,062.1490	3,223.78658	-9,863.7442	3,739.4461

Q8NDM7	0.3565804	1.000000	4,135.5448	4,363.95731	-5,071.6003	13,342.6899
P69905	0.3593239	1.000000	-1,176.4338	1,248.69842	-3,810.9571	1,458.0896
P07737	0.3635745	1.000000	-1,530.5128	1,639.34018	-4,989.2183	1,928.1926
P09871	0.3659998	1.000000	13,868.6927	14,932.02491	-17,635.1260	45,372.5115
Q9ULZ9	0.3682786	1.000000	1,748.3116	1,891.55334	-2,242.5171	5,739.1403
P63104	0.3704819	1.000000	-187.3309	203.63714	-616.9677	242.3059
P06681	0.3725003	1.000000	-10,592.8982	11,564.83748	-34,992.5725	13,806.7760
P22314	0.3745666	1.000000	-586.0287	642.63842	-1,941.8772	769.8199
O75636	0.3758650	1.000000	2,836.4503	3,119.11982	-3,744.3173	9,417.2179
Q04756	0.3770092	1.000000	-791.7860	872.83179	-2,633.3001	1,049.7281
Q96S96	0.3772403	1.000000	200.3257	220.94019	-265.8174	666.4687
P00747	0.3791001	1.000000	24,595.5005	27,235.00455	-32,865.3364	82,056.3373
P61769	0.3867835	1.000000	-711.9546	801.49323	-2,402.9575	979.0483
P04080	0.3930002	1.000000	1,578.1544	1,585.81344	-3,468.4447	6,624.7536
Q9NUJ3	0.3952655	1.000000	199.0629	228.23783	-282.4768	680.6027
P02760	0.3959648	1.000000	-28,317.8254	32,517.25878	-96,923.2445	40,287.5937
P04180	0.4003870	1.000000	-82.2452	95.35071	-283.4177	118.9272
Q9NQ76	0.4011966	1.000000	-197.1777	228.99886	-680.3230	285.9677
P01591	0.4013917	1.000000	-2,071.4170	2,406.72816	-7,149.1696	3,006.3355
P02746	0.4015011	1.000000	-3,816.3813	4,435.21110	-13,173.8588	5,541.0962
Q8NCS4	0.4037028	1.000000	-230.3802	269.01923	-797.9612	337.2007
P07359	0.4047257	1.000000	-315.6752	369.43992	-1,095.1253	463.7748
P02765	0.4062443	1.000000	38,133.4949	44,775.84918	-56,335.2892	132,602.2790

Q06830	0.4096699	1.000000	516.9914	541.63364	-1,197.9708	2,231.9537
Q9Y2K3	0.4130205	1.000000	-1,701.4319	2,027.52255	-5,979.1305	2,576.2668
Q06033	0.4150232	1.000000	-2,593.0525	3,103.58151	-9,141.0371	3,954.9321
Q9NPH3	0.4154863	1.000000	-5,569.2523	6,672.50021	-19,646.9972	8,508.4926
P19652	0.4223802	1.000000	-218.3490	265.58682	-778.6882	341.9902
Q5VYS8	0.4245992	1.000000	1,604.4429	1,961.09546	-2,533.1068	5,741.9926
P27169	0.4260116	1.000000	-3,875.1344	4,751.29299	-13,899.4864	6,149.2175
Q9Y4P3	0.4331961	1.000000	-76.0891	94.78485	-276.0677	123.8894
Q86X10	0.4366511	1.000000	-2,511.4950	3,152.65185	-9,163.0090	4,140.0190
Q76LX8	0.4387567	1.000000	154.0952	194.34123	-255.9290	564.1193
P38570	0.4402279	1.000000	-3,130.5648	3,961.14329	-11,487.8466	5,226.7170
Q9NWU2	0.4445266	1.000000	803.0700	915.36140	-2,099.6830	3,705.8230
P05452	0.4449133	1.000000	-8,311.6170	10,627.30592	-30,733.2726	14,110.0385
P0C0L4	0.4460865	1.000000	14,857.0271	19,046.23170	-25,327.0092	55,041.0634
P19320	0.4478564	1.000000	657.9929	846.87557	-1,128.7583	2,444.7442
Q5VUB5	0.4509583	1.000000	687.9476	891.61348	-1,193.1924	2,569.0876
Q5T8A7	0.4537012	1.000000	499.7427	651.69993	-875.2239	1,874.7094
P55899	0.4598306	1.000000	50,573.0648	59,973.80139	-138,054.3419	239,200.4715
P07437	0.4623863	1.000000	101.3198	134.74894	-182.9756	385.6152
O75530	0.4664773	1.000000	341.2102	458.03158	-625.1519	1,307.5724
O75882	0.4760456	1.000000	1,567.3192	2,150.54997	-2,969.9446	6,104.5830
P06276	0.4792912	1.000000	-5,161.1605	7,134.92901	-20,214.5449	9,892.2239
P20742	0.4829144	1.000000	822.8877	1,147.16133	-1,597.4112	3,243.1865

Q9H6J7	0.4890830	1.000000	-325.5717	460.43057	-1,296.9953	645.8518
Q2TV78	0.4906221	1.000000	-72.3894	102.74350	-289.1592	144.3805
P22792	0.4932192	1.000000	-1,891.6720	2,701.26671	-7,590.8466	3,807.5026
P0DJI8	0.5004096	1.000000	-367.8885	534.31185	-1,495.1880	759.4110
P06396	0.5006039	1.000000	-21,598.2787	31,383.21575	-87,811.0762	44,614.5188
Q03591	0.5036644	1.000000	3,464.7562	5,071.10369	-7,234.3374	14,163.8497
Q15165	0.5098445	1.000000	131.7724	195.72884	-281.1793	544.7242
Q9UJJ9	0.5110670	1.000000	-42.3274	63.05570	-175.3633	90.7084
P22891	0.5118123	1.000000	155.6994	232.36234	-334.5423	645.9411
Q9Y2I7	0.5132766	1.000000	407.6506	610.51232	-880.4179	1,695.7190
P02788	0.5143716	1.000000	-415.5010	623.91119	-1,731.8385	900.8365
P35858	0.5144198	1.000000	3,346.1715	5,025.15343	-7,255.9755	13,948.3185
Q01082	0.5152331	1.000000	-4,430.1822	6,666.12931	-18,494.4857	9,634.1213
P00748	0.5168728	1.000000	3,739.0567	5,648.49533	-8,178.2267	15,656.3401
P02042	0.5247391	1.000000	11.2890	17.38287	-25.3857	47.9636
P05543	0.5272432	1.000000	-1,410.8465	2,185.78667	-6,022.4533	3,200.7602
P07996	0.5317456	1.000000	117.5559	184.15292	-270.9728	506.0846
Q96M86	0.5333122	1.000000	-708.7606	1,114.58572	-3,060.3309	1,642.8097
Q14508	0.5374299	1.000000	-10,536.9662	16,740.25876	-45,855.8249	24,781.8925
P19823	0.5414046	1.000000	-11,950.4360	19,174.97002	-52,406.0865	28,505.2144
P22105	0.5450784	1.000000	1,029.9383	1,667.87461	-2,488.9695	4,548.8462
P20472	0.5451426	1.000000	-11.6039	18.79428	-51.2564	28.0486
P13671	0.5471018	1.000000	4,158.1172	6,768.02808	-10,121.1738	18,437.4083

P12830	0.5503826	1.000000	-133.0249	218.32412	-593.6485	327.5987
P80723	0.5591858	1.000000	-10,972.4622	18,417.64513	-49,830.2968	27,885.3724
P19827	0.5603602	1.000000	14,856.5463	25,012.69710	-37,915.6317	67,628.7243
P17661	0.5609521	1.000000	453.6314	764.90640	-1,160.1800	2,067.4429
Q6ZMB0	0.5614065	1.000000	-190.6831	321.90397	-869.8411	488.4749
O00187	0.5624886	1.000000	-2,793.7421	4,729.48995	-12,772.0937	7,184.6095
P02774	0.5648732	1.000000	-22,755.9917	38,762.01403	-104,536.6927	59,024.7094
Q5HYC2	0.5793627	1.000000	-520.6184	921.22447	-2,464.2322	1,422.9953
P11142	0.5894423	1.000000	-161.8589	294.26419	-782.7020	458.9843
P08151	0.5902086	1.000000	2,066.1423	3,764.12134	-5,875.4596	10,007.7441
Q6UXB8	0.5928930	1.000000	-9,703.1935	17,806.97518	-47,272.6272	27,866.2401
P01008	0.5951261	1.000000	-5,036.0573	9,298.59084	-24,654.3691	14,582.2546
A1KZ92	0.5957372	1.000000	-15,923.7952	29,451.02590	-78,060.0284	46,212.4381
P01700	0.6081458	1.000000	-2,429.6832	4,651.16932	-12,242.7927	7,383.4263
P68363	0.6089907	1.000000	67.8559	130.20632	-206.8554	342.5672
P0DP02	0.6112698	1.000000	319.1655	616.38615	-981.2956	1,619.6266
P36980	0.6127481	1.000000	3,795.3789	7,360.55053	-11,734.0252	19,324.7831
P15812	0.6131263	1.000000	-179.9110	349.28414	-916.8361	557.0141
B1AKI9	0.6137687	1.000000	-11,078.8764	21,548.11741	-56,541.4301	34,383.6774
Q8IYX8	0.6146634	1.000000	-437.4952	853.08517	-2,237.3476	1,362.3572
P32119	0.6174437	1.000000	-409.3251	804.51793	-2,106.7096	1,288.0593
P18206	0.6191065	1.000000	-601.4618	1,187.81066	-3,107.5232	1,904.5997
Q5VU43	0.6248615	1.000000	-4,136.8954	8,306.93939	-21,663.0056	13,389.2147

O60318	0.6255527	1.000000	260.9886	525.12417	-846.9265	1,368.9037
P08294	0.6285910	1.000000	-293.2869	595.37519	-1,549.4187	962.8450
P02775	0.6291298	1.000000	-1,291.4789	2,625.86402	-6,831.5677	4,248.6099
Q06828	0.6363333	1.000000	-14,813.2802	30,767.83973	-79,727.7478	50,101.1873
Q5JRA6	0.6387405	1.000000	-34.6437	72.47676	-187.5563	118.2689
Q9ULU4	0.6433130	1.000000	-504.4805	1,070.06070	-2,762.1112	1,753.1502
Q13936	0.6448414	1.000000	-86.5976	184.53816	-475.9391	302.7439
Q9NQ79	0.6470120	1.000000	-277.7308	595.77269	-1,534.7013	979.2397
Q6IPM2	0.6475514	1.000000	-482.5314	1,036.80972	-2,670.0087	1,704.9459
O95497	0.6517204	1.000000	-1,318.0578	2,868.67169	-7,370.4261	4,734.3104
P12259	0.6518443	1.000000	-13,022.1053	28,352.67656	-72,841.0240	46,796.8133
Q8N9M5	0.6523827	1.000000	-353.4967	770.94286	-1,980.0439	1,273.0506
Q15166	0.6545987	1.000000	1,624.5405	3,567.44409	-5,902.1086	9,151.1896
Q00059	0.6553494	1.000000	-511.8012	1,126.53417	-2,888.5805	1,864.9782
P43251	0.6571880	1.000000	437.2647	968.02153	-1,605.0822	2,479.6116
P55290	0.6575855	1.000000	-410.4436	909.77843	-2,329.9083	1,509.0211
P0CF74	0.6601488	1.000000	-205.2330	458.59969	-1,172.7938	762.3277
P07195	0.6666742	1.000000	-33.6371	76.74113	-195.5467	128.2726
Q9Y6J0	0.6687348	1.000000	-570.2373	1,309.62303	-3,333.3003	2,192.8258
P11226	0.6735149	1.000000	160.3523	374.03144	-628.7851	949.4896
Q92503	0.6750230	1.000000	-334.8151	784.84305	-1,990.6892	1,321.0590
Q13627	0.6792942	1.000000	-1,266.3355	3,010.56521	-7,618.0729	5,085.4018
P36955	0.6841951	1.000000	1,611.1953	3,893.67026	-6,603.7308	9,826.1215

P19838	0.6901305	1.000000	-63.5395	156.67413	-394.0930	267.0141
P05155	0.6930472	1.000000	-2,939.5591	7,321.26579	-18,386.0797	12,506.9615
P35579	0.6948291	1.000000	-1,937.2222	4,854.66188	-12,179.6635	8,305.2190
Q5T013	0.6959593	1.000000	84.1575	211.72722	-362.5479	530.8629
Q9Y5Y7	0.6974761	1.000000	-84.1084	212.72512	-532.9191	364.7024
Q9UNN8	0.7019852	1.000000	119.8400	307.94085	-529.8584	769.5384
P00450	0.7060761	1.000000	-25,363.5515	66,130.70530	-164,887.1437	114,160.0407
O14556	0.7074623	1.000000	193.7867	507.78392	-877.5438	1,265.1171
P41594	0.7130574	1.000000	-37.6603	100.70695	-250.1334	174.8128
Q16513	0.7143410	1.000000	-557.3635	1,497.47212	-3,716.7535	2,602.0265
P07360	0.7146199	1.000000	-1,979.4268	5,323.59743	-13,211.2356	9,252.3820
P06576	0.7146267	1.000000	2,384.5905	6,413.42971	-11,146.5634	15,915.7445
Q96B67	0.7186836	1.000000	367.6437	1,003.75615	-1,750.0967	2,485.3840
P0DOX5	0.7188822	1.000000	449.0692	1,226.97537	-2,139.6225	3,037.7610
O95045	0.7203983	1.000000	41.1156	112.97721	-197.2455	279.4767
A0A075B6N3	0.7270369	1.000000	-81.2384	228.91190	-564.2003	401.7235
Q13617	0.7285146	1.000000	221.4112	627.43733	-1,102.3658	1,545.1882
Q9NP66	0.7297091	1.000000	-34.2158	97.40877	-239.7303	171.2987
P22352	0.7315029	1.000000	474.4972	1,360.26927	-2,395.4201	3,344.4145
P07357	0.7323972	1.000000	-1,132.0165	3,256.53827	-8,002.7117	5,738.6787
Q04727	0.7336934	1.000000	-1.7060	4.93273	-12.1132	8.7011
P26927	0.7341574	1.000000	515.1585	1,492.22563	-2,633.1623	3,663.4794
Q15113	0.7341854	1.000000	-826.8326	2,395.29433	-5,880.4619	4,226.7967

P18428	0.7405972	1.000000	-240.4501	714.51956	-1,747.9546	1,267.0544
Q8NEP9	0.7422524	1.000000	-890.1443	2,662.82229	-6,508.2083	4,727.9197
Q86Y33	0.7426137	1.000000	-575.9499	1,725.44131	-4,216.3129	3,064.4130
P02743	0.7450972	1.000000	-9,693.4074	29,333.90601	-71,582.5393	52,195.7244
Q16610	0.7473930	1.000000	-2,542.9020	7,767.92937	-18,931.8004	13,845.9964
Q9Y4R8	0.7476533	1.000000	-353.4897	1,080.97922	-2,634.1565	1,927.1771
Q9NZJ4	0.7505304	1.000000	1,990.8764	6,161.04269	-11,007.7874	14,989.5403
Q15848	0.7513742	1.000000	-402.1814	1,248.98820	-3,037.3161	2,232.9534
A2CJ06	0.7528260	1.000000	-183.4011	573.02819	-1,392.3849	1,025.5827
Q6ZN68	0.7535164	1.000000	14.0763	44.10836	-78.9842	107.1368
P08603	0.7569700	1.000000	8,168.6083	25,973.49991	-46,630.6864	62,967.9030
Q86YV5	0.7582401	1.000000	-1,001.5775	3,202.01258	-7,757.2335	5,754.0785
P0DOX2	0.7612017	1.000000	-98.9491	320.39734	-774.9284	577.0302
Q70CQ4	0.7625135	1.000000	-915.6051	2,981.66714	-7,206.3729	5,375.1626
Q14624	0.7634848	1.000000	8,067.0771	26,381.94875	-47,593.9693	63,728.1236
P07358	0.7666503	1.000000	2,227.1709	7,385.66735	-13,355.2251	17,809.5669
P43121	0.7680048	1.000000	3,395.8611	11,329.12282	-20,506.4987	27,298.2209
Q16512	0.7729844	1.000000	18.7273	63.89133	-116.0716	153.5262
P50748	0.7736927	1.000000	-87.9382	300.98358	-722.9580	547.0817
P61626	0.7789152	1.000000	199.1656	698.26761	-1,274.0503	1,672.3815
O43866	0.7789567	1.000000	592.9769	2,079.35798	-3,794.0849	4,980.0388
O15195	0.7793103	1.000000	14.4553	50.77301	-92.6664	121.5769
B9A064	0.7804935	1.000000	-479.9957	1,695.29908	-4,056.7641	3,096.7727

Q86YA3	0.7834179	1.000000	101.0558	361.87598	-662.4358	864.5473
Q8NHU2	0.7861103	1.000000	73.8268	267.79053	-491.1618	638.8155
P01344	0.7866453	1.000000	-161.4991	587.31108	-1,400.6171	1,077.6190
P27918	0.7871919	1.000000	353.7230	1,289.75044	-2,367.4125	3,074.8586
Q9NPJ6	0.7879522	1.000000	-303.0630	1,109.10150	-2,643.0626	2,036.9367
P05156	0.7881378	1.000000	-1,335.1893	4,890.70773	-11,653.6806	8,983.3021
Q9Y6R7	0.7905028	1.000000	-147.7323	547.40395	-1,302.6537	1,007.1891
P25311	0.7968666	1.000000	-3,518.9020	13,457.81122	-31,912.4018	24,874.5977
Q96SN8	0.7992639	1.000000	231.3608	895.64791	-1,658.2911	2,121.0128
P00915	0.7998988	1.000000	-745.4524	2,895.18227	-6,853.7531	5,362.8483
P00746	0.8049255	1.000000	-318.8936	1,271.17606	-3,000.8406	2,363.0534
P00918	0.8078557	1.000000	137.3373	555.98993	-1,035.6989	1,310.3736
Q96HW7	0.8127363	1.000000	-457.2502	1,900.39181	-4,466.7264	3,552.2260
P00751	0.8143817	1.000000	-6,769.2913	28,388.56879	-66,663.9360	53,125.3534
Q9UNW1	0.8154020	1.000000	42.1308	177.68122	-332.7439	417.0054
P02745	0.8166672	1.000000	-810.8419	3,443.69520	-8,076.4037	6,454.7199
Q6ZTR5	0.8175149	1.000000	62.1790	265.32885	-497.6160	621.9739
P22626	0.8182786	1.000000	-159.5624	683.79906	-1,602.2523	1,283.1275
P51884	0.8195209	1.000000	1,945.8508	8,397.38404	-15,771.0809	19,662.7824
Q96IY4	0.8196234	1.000000	898.8547	3,881.28370	-7,289.9381	9,087.6475
Q96AE4	0.8201149	1.000000	-182.6203	790.75588	-1,850.9694	1,485.7288
Q14520	0.8286537	1.000000	2,594.4764	11,804.44711	-22,310.7300	27,499.6828
P68871	0.8303038	1.000000	1,579.2572	7,256.42527	-13,730.4619	16,888.9763

Q6IA86	0.8307130	1.000000	62.4757	287.77096	-544.6680	669.6193
P23142	0.8382396	1.000000	541.0161	2,609.84106	-4,965.2672	6,047.2994
P14625	0.8388666	1.000000	148.9299	721.27047	-1,372.8177	1,670.6776
Q9NRC6	0.8399523	1.000000	261.7322	1,276.30317	-2,431.0321	2,954.4965
Q8IVW1	0.8472541	1.000000	-40.3815	206.46547	-475.9855	395.2226
Q709C8	0.8483651	1.000000	-1,125.7996	5,798.82156	-13,360.2436	11,108.6445
P17936	0.8485166	1.000000	-96.2811	496.43210	-1,143.6613	951.0990
P31937	0.8507654	1.000000	575.7066	3,013.70703	-5,782.6595	6,934.0726
P05160	0.8519412	1.000000	1,206.1731	6,364.86075	-12,222.5093	14,634.8554
O95445	0.8541267	1.000000	-967.6090	5,183.44609	-11,903.7243	9,968.5063
P07203	0.8607553	1.000000	-25.9233	145.56100	-333.0302	281.1835
Q12805	0.8632638	1.000000	-294.8440	1,686.27731	-3,852.5781	3,262.8902
Q92945	0.8846146	1.000000	211.6614	1,436.77152	-2,819.6615	3,242.9844
Q5SWL8	0.8867661	1.000000	42.3218	292.78357	-575.3975	660.0412
P13591	0.8870644	1.000000	-20.7399	143.86108	-324.2603	282.7804
Q9UKI2	0.8875318	1.000000	-228.1290	1,589.02674	-3,580.6824	3,124.4244
P08238	0.8894039	1.000000	-56.4938	400.21495	-900.8735	787.8860
Q7Z406	0.8940039	1.000000	77.5148	573.12819	-1,131.6800	1,286.7095
Q9UGM5	0.8953631	1.000000	522.2303	3,911.74435	-7,730.8289	8,775.2895
Q15582	0.8981325	1.000000	77.1359	593.58863	-1,175.2267	1,329.4984
Q8TDG4	0.8985618	1.000000	-31.0830	240.21339	-537.8890	475.7229
P15169	0.8998519	1.000000	-203.3394	1,591.79435	-3,561.7320	3,155.0531
P06727	0.9053654	1.000000	1,729.0864	14,328.78631	-28,502.0102	31,960.1829

Q96PD5	0.9059832	1.000000	1,536.7424	12,818.95032	-25,508.8787	28,582.3635
Q96JB1	0.9060779	1.000000	122.5393	1,023.21419	-2,036.2540	2,281.3325
Q9BXR6	0.9068268	1.000000	136.5616	1,149.51431	-2,288.7016	2,561.8248
P23443	0.9084783	1.000000	-12.1271	103.93157	-231.4035	207.1494
P20138	0.9090049	1.000000	-7.2479	62.47734	-139.0636	124.5678
P02747	0.9107283	1.000000	-512.9566	4,507.46431	-10,022.8751	8,996.9618
P22692	0.9157136	1.000000	-510.4154	4,751.59978	-10,535.4146	9,514.5839
P15144	0.9224937	1.000000	77.9031	788.91139	-1,586.5544	1,742.3606
P05362	0.9247727	1.000000	16.1098	168.10053	-338.5513	370.7709
P02749	0.9267190	1.000000	-2,482.6728	26,596.08440	-58,595.5060	53,630.1604
P04278	0.9326586	1.000000	-78.3342	913.40329	-2,005.4466	1,848.7783
P00734	0.9445428	1.000000	1,482.5764	21,000.72590	-42,825.0822	45,790.2351
P05090	0.9447658	1.000000	15.2354	216.68256	-441.9248	472.3956
P05546	0.9450208	1.000000	709.4320	10,136.64066	-20,677.0104	22,095.8743
Q9UHR6	0.9451479	1.000000	65.6564	940.30162	-1,918.2066	2,049.5194
P01019	0.9473218	1.000000	860.3196	12,830.43685	-26,209.5359	27,930.1752
P33151	0.9473694	1.000000	-129.9351	1,939.55055	-4,222.0290	3,962.1589
P02656	0.9500831	1.000000	-96.2122	1,514.36477	-3,291.2425	3,098.8182
P05109	0.9502136	1.000000	19.0767	301.05191	-616.0873	654.2407
Q9NUB1	0.9550295	1.000000	-53.4369	933.72666	-2,023.4279	1,916.5542
P02649	0.9553923	1.000000	273.4495	4,817.01216	-9,889.5578	10,436.4568
P09172	0.9598384	1.000000	-11.8113	231.12368	-499.4397	475.8170
P04004	0.9601104	1.000000	685.3724	13,502.87365	-27,803.2008	29,173.9455

P62937	0.9664998	1.000000	-6.6043	154.95262	-333.5258	320.3171
P48740	0.9701092	1.000000	-60.7366	1,597.19963	-3,430.5333	3,309.0601
P01011	0.9734497	1.000000	1,376.3974	40,751.53163	-84,601.8189	87,354.6136
P07197	0.9736117	1.000000	144.4255	4,302.32111	-8,932.6786	9,221.5296
O00391	0.9767092	1.000000	-34.9503	1,179.66012	-2,523.8156	2,453.9150
Q9ULB1	0.9801597	1.000000	-10.2302	405.36343	-865.4723	845.0119
Q0JRZ9	0.9817451	1.000000	-111.3138	4,793.85160	-10,225.4566	10,002.8290
P41222	0.9848957	1.000000	-19.7477	1,027.88752	-2,188.4009	2,148.9054
P08697	0.9852533	1.000000	-101.7779	5,426.10405	-11,549.8567	11,346.3010
Q9NWQ4	0.9886595	1.000000	-2.4026	166.56641	-353.8270	349.0218
O95294	0.9925987	1.000000	7.2026	765.11602	-1,607.0511	1,621.4562
P00742	0.9935659	1.000000	34.4228	4,206.37515	-8,840.2530	8,909.0986
A6NHR8	0.9939737	1.000000	-17.7348	2,313.83061	-4,899.4907	4,864.0211
P42702	0.9962983	1.000000	-7.1375	1,516.02456	-3,205.6698	3,191.3947
P02790	0.9969002	1.000000	268.1562	68,015.50518	-143,232.0161	143,768.3286
P02750	0.9999514	1.000000	-0.3148	5,089.54581	-10,738.3178	10,737.6882

Supplementary table 4: Recurrent depression related to LLD patients – Independent sample t-test

Accession	Sig. (2-tailed)	Benjamini-Hochberg Adjusted P value	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
					Lower	Upper
P13671	0.0257140	1	-11879.7911	4860.372208	-22134.2801	-1625.302102
P05156	0.0262610	1	-8482.700255	3485.284235	-15836.00723	-1129.393283
P06276	0.0288770	1	-12356.29002	5176.558856	-23277.87453	-1434.705506
Q14624	0.0352800	1	-43688.79689	19101.91955	-83990.32432	-3387.26946

Q8NDM7	0.0371890	1	-7329.09813	3242.039808	-14169.20422	-488.9920385
O76027	0.0388900	1	516.5878761	230.8171984	29.6061553	1003.569597
O60318	0.0394460	1	-856.9391711	384.1226054	-1667.367028	-46.5113145
A0A0A0MS15	0.0395800	1	-13352.71441	5989.93012	-25990.36229	-715.0665306
P09172	0.0401800	1	-373.2709414	168.0211612	-727.7646046	-18.7772782
P03952	0.0448830	1	-4998.310755	2308.423355	-9868.658309	-127.9632003
P14625	0.0554750	1	-1097.170127	533.6495738	-2223.072311	28.7320568
Q9Y4R8	0.0582210	1	1631.205166	803.2481817	-63.500361	3325.910692
Q9ULU4	0.0669450	1	-1572.461337	803.4054669	-3267.498707	122.5760324
Q8N9M5	0.0712710	1	1116.708192	580.4284985	-107.8888964	2341.30528
Q562R1	0.0730160	1	529.3999863	277.0263957	-55.074619	1113.874592
Q9NQ79	0.0767690	1	-848.3987281	450.2905994	-1798.428849	101.631393
P15812	0.0796490	1	493.7284981	264.84016	-65.0353972	1052.492393
Q2TV78	0.0819310	1	-145.2036136	78.5325163	-310.8927398	20.4855127
P08603	0.0864910	1	-35804.74115	19678.30394	-77322.33335	5712.85104
O14788	0.0866110	1	-577.3606666	317.4495378	-1247.120647	92.3993134
Q9UHR6	0.0897430	1	-1280.612484	711.7200287	-2782.210488	220.9855192
P35579	0.0903850	1	6629.474451	3692.443558	-1160.900488	14419.84939
Q15165	0.0992820	1	-262.9814429	150.8261554	-581.1968151	55.2339293
P41146	0.1003050	1	-8728.491664	5022.394598	-19324.81803	1867.834697
P00751	0.1008980	1	-37542.50283	21642.88077	-83204.98984	8119.984168
P07357	0.1042130	1	-4276.510038	2491.246525	-9532.580764	979.5606875
P01008	0.1048760	1	-12250.72838	7151.329738	-27338.71526	2837.258508

Q13936	0.1103600	1	-239.1440591	141.9701424	-538.6748772	60.3867589
P43251	0.1182420	1	1228.853354	746.8256959	-346.8111336	2804.517841
P00736	0.1199740	1	-7426.072882	4535.959995	-16996.11194	2143.966175
P04004	0.1213710	1	-16907.36232	10369.08292	-38784.215	4969.490356
P02747	0.1260870	1	-5580.131303	3468.655915	-12898.35559	1738.09298
P02760	0.1310340	1	-40635.07298	25611.85895	-94671.37198	13401.22601
P61626	0.1346860	1	848.3022863	540.0924254	-291.1931263	1987.797699
Q9UGM5	0.1349280	1	-4740.658588	3020.257362	-11112.84462	1631.527444
P47895	0.1398900	1	-1264.427197	816.4921017	-2987.074952	458.2205585
Q9NZJ4	0.1409390	1	-7379.557665	4778.736506	-17461.81039	2702.695058
Q9NRC6	0.1485030	1	1499.258579	990.5417294	-590.6017919	3589.118951
Q9Y2M0	0.1488580	1	-3118.050897	2061.976247	-7468.440505	1232.338711
Q8IYW2	0.1514490	1	-10532.90629	7012.683902	-25328.37603	4262.563443
P19827	0.1573750	1	-29053.23748	19641.00876	-70492.14373	12385.66878
P05452	0.1575030	1	-12432.17691	8407.335751	-30170.10485	5305.751022
P07225	0.1625610	1	-9894.056196	6777.397291	-24193.11458	4405.002186
Q9Y2K3	0.1631010	1	2348.303089	1610.767259	-1050.118765	5746.724944
P08238	0.1646630	1	452.903098	311.8817683	-205.1099153	1110.916111
Q5VYS8	0.1647320	1	-2260.871458	1557.166463	-5546.20552	1024.462603
Q5T8A7	0.1647380	1	-749.5774869	516.2774128	-1838.827615	339.672641
Q7Z406	0.1699640	1	640.9593674	447.2532064	-302.6624147	1584.58115
Q76LX8	0.1719900	1	220.2186012	154.4373392	-105.6157028	546.0529052
P00742	0.1738560	1	4661.556406	3284.146544	-2267.387133	11590.49994

P02745	0.1776630	1	-3790.842173	2695.691576	-9478.254254	1896.569908
P01042	0.1791420	1	-22060.31676	15743.731	-55276.68567	11156.05215
P15144	0.1796010	1	-863.6229742	617.0259259	-2165.433885	438.1879362
P13591	0.1814890	1	-156.8914101	112.606539	-394.4704402	80.68762
P05109	0.1817690	1	327.9591001	235.5470675	-169.0017723	824.9199726
P04217	0.1884970	1	-63251.05074	46167.19287	-160655.3134	34153.21196
P09871	0.1915340	1	-16325.62333	12002.41808	-41648.51197	8997.265308
P02656	0.1961690	1	-1599.544344	1188.899853	-4107.903773	908.8150859
Q5SWL8	0.1975550	1	-308.4966084	230.0446884	-793.8484756	176.8552588
O15195	0.2009400	1	-53.2098684	39.9936473	-137.5890886	31.1693517
P80723	0.2018520	1	-19418.30563	14626.2558	-50277.00796	11440.3967
P80108	0.2031950	1	-702.3862788	530.7056796	-1822.077389	417.3048313
Q96IY4	0.2042190	1	-4036.314838	3056.994819	-10486.01013	2413.380453
Q9UKI2	0.2049110	1	1648.496757	1250.530638	-989.8922633	4286.885777
O95445	0.2059460	1	5367.994765	4081.874254	-3244.007123	13979.99665
P0DJI8	0.2096100	1	-556.1376914	426.4813827	-1455.934756	343.6593734
P22352	0.2125420	1	-1392.931026	1075.40112	-3661.829062	875.9670101
P02751	0.2228820	1	-40777.84367	32231.40147	-108780.1566	27224.46925
A0A075B6N3	0.2230700	1	229.3732505	181.3769169	-153.2985942	612.0450953
O60308	0.2246120	1	-6058.98477	4807.844086	-16202.64912	4084.679578
P11142	0.2249440	1	295.2301199	234.4423695	-199.4000434	789.8602833
Q15063	0.2260100	1	-1023.524979	814.7367816	-2742.469333	695.4193743
Q9NPH3	0.2342960	1	-6641.394944	5385.672671	-18004.17104	4721.381155

P02100	0.2349970	1	-2068.682726	1680.169481	-5613.53047	1476.165017
Q63HN8	0.2374700	1	22326.72548	18233.66218	-16142.93903	60796.38999
P15559	0.2399460	1	609.5808254	500.5724378	-446.5347017	1665.696353
P01700	0.2455000	1	4470.400205	3716.347317	-3370.407257	12311.20767
P27918	0.2455940	1	-1232.277151	1024.631115	-3394.059838	929.5055362
Q14520	0.2473040	1	-11230.45284	9373.308017	-31006.40411	8545.498428
P85298	0.2486960	1	-6024.043046	5043.297125	-16664.46988	4616.383793
P18206	0.2520630	1	1126.0075	949.684318	-877.6512683	3129.666268
Q08380	0.2565340	1	-776.6710463	661.4922492	-2172.297698	618.9556056
Q9Y5Y7	0.2571670	1	-199.0059821	169.7284184	-557.1016434	159.0896791
P19823	0.2581960	1	-18021.74789	15404.9809	-50523.41658	14479.9208
P06681	0.2600530	1	-10968.03066	9413.50817	-30828.79684	8892.735522
P04080	0.2603010	1	779.1229336	669.0580233	-632.4661065	2190.711974
Q9NWU2	0.2618750	1	-447.1285942	385.2821155	-1260.002803	365.7456149
P05155	0.2654030	1	6737.193015	5850.014403	-5605.258504	19079.64453
P10643	0.2656430	1	-10176.7018	8841.197218	-28829.99742	8476.59382
Q86Y33	0.2697260	1	1571.747816	1377.628589	-1334.794442	4478.290074
P22792	0.2726460	1	-2473.188457	2181.475526	-7075.699503	2129.32259
Q13561	0.2775380	1	927.7229236	826.9853061	-817.0635579	2672.509405
P00740	0.2775390	1	-1862.095504	1659.901771	-5364.182119	1639.99111
Q709C8	0.2801070	1	5162.122734	4627.13476	-4600.278263	14924.52373
P23142	0.2823680	1	-2313.131979	2083.517993	-6708.970697	2082.706738
P51884	0.2830030	1	7435.56449	6706.62183	-6714.170722	21585.2997

P07197	0.2858750	1	3781.829739	3432.194717	-3459.468141	11023.12762
P08519	0.2867240	1	-1337.450734	1216.016975	-3903.022291	1228.120824
Q92945	0.2876780	1	-1259.15845	1147.178719	-3679.493981	1161.177081
Q9H254	0.2880110	1	107.3661236	97.887814	-99.1591112	313.8913584
P07203	0.2957430	1	125.5594436	116.3870992	-119.9958713	371.1147586
Q15848	0.2972270	1	1076.434468	1000.971907	-1035.431655	3188.300592
P49908	0.3016210	1	-215.2024157	202.0040743	-641.3937584	210.9889271
Q14508	0.3037680	1	14361.02701	13542.2099	-14210.53839	42932.59241
Q86YV5	0.3059710	1	-2711.280682	2568.739457	-8130.847204	2708.28584
P08151	0.3064240	1	3203.243886	3037.771399	-3205.893534	9612.381306
Q9NPJ6	0.3076250	1	935.3850629	889.3396842	-940.9576569	2811.727783
Q06830	0.3091510	1	-246.3010466	234.9399966	-741.9811114	249.3790181
Q9UNW1	0.3101610	1	-149.0116738	142.4446012	-449.5435123	151.5201647
Q15361	0.3108580	1	627.5267356	600.7627425	-639.9718571	1895.025328
P17936	0.3109390	1	-415.4553565	397.8055181	-1254.751636	423.8409225
P22692	0.3156540	1	-3936.135429	3806.963757	-11968.12687	4095.85601
P02753	0.3169260	1	-21383.41744	20737.76649	-65136.28023	22369.44535
P55290	0.3176950	1	-754.7913241	733.2004862	-2301.709132	792.1264833
P06396	0.3183420	1	26206.45596	25491.9118	-27576.77666	79989.68858
Q16610	0.3190960	1	-6408.504236	6243.791397	-19581.75259	6764.744119
Q9H6J7	0.3196140	1	-383.7909213	374.3390363	-1173.577252	405.9954089
A1KZ92	0.3200730	1	-24380.32941	23803.12774	-74600.53912	25839.88029
P0DOX2	0.3232060	1	262.066588	257.574116	-281.3672943	805.5004703

Q9ULZ9	0.3260440	1	-1572.170998	1554.581294	-4852.05083	1707.708834
P01344	0.3313310	1	-472.341199	472.33955	-1468.89054	524.2081416
P07359	0.3315410	1	302.6329688	302.7668627	-336.1492745	941.415212
Q13627	0.3317850	1	-2426.187142	2428.521962	-7549.920609	2697.546326
P00746	0.3332420	1	-1018.039355	1022.181909	-3174.654671	1138.57596
P00488	0.3517330	1	-618.2577815	645.6979875	-1980.561454	744.0458911
P50402	0.3523220	1	-562.6532512	588.3627305	-1803.990105	678.683603
P08670	0.3573070	1	128.4641321	135.7697371	-157.9849742	414.9132384
Q96AE4	0.3605980	1	599.0904152	637.6226683	-746.1758231	1944.356654
Q9UNN8	0.3619410	1	-233.3346536	249.0552382	-758.795275	292.1259677
Q9BYX7	0.3634160	1	-681.883014	730.1203092	-2222.302216	858.5361881
P0CG47	0.3684990	1	26.2498568	28.4139572	-33.6983528	86.1980664
Q5HYC2	0.3688920	1	-691.5957435	749.2417834	-2272.35773	889.1662427
P49747	0.3696680	1	-9481.355549	10288.7222	-31188.66192	12225.95082
P12259	0.3753820	1	-20939.41056	23002.29416	-69470.0091	27591.18799
O00187	0.3787200	1	-3482.98599	3853.621081	-11613.41578	4647.443798
P31937	0.3822600	1	-2183.430205	2434.219695	-7319.184838	2952.324428
P01591	0.3840830	1	1772.700184	1984.077048	-2413.336478	5958.736847
P04180	0.3865360	1	69.8846167	78.6316427	-96.013648	235.7828813
P05543	0.3889170	1	-1579.398958	1786.22361	-5348.001356	2189.20344
P08185	0.3929900	1	-309.5674443	353.1969869	-1054.747949	435.6130607
Q6S8J3	0.3960520	1	-130.2792258	149.6273818	-445.9654067	185.4069551
O95497	0.3999520	1	-2014.063895	2332.795069	-6935.831272	2907.703482

Q9C056	0.4017450	1	72.5445342	84.3523535	-105.4233752	250.5124436
P07996	0.4076250	1	127.9661893	150.7083911	-190.000722	445.9331007
P33151	0.4088090	1	1328.791708	1568.985409	-1981.478148	4639.061564
Q9UJJ9	0.4094130	1	-43.7074041	51.6759809	-152.7341935	65.3193854
P07360	0.4104730	1	-3648.808236	4324.023235	-12771.69982	5474.083344
P20138	0.4137920	1	-42.3702517	50.5759474	-149.0761734	64.33567
Q5T013	0.4148070	1	-143.8887406	172.1365896	-507.0651989	219.2877176
P02788	0.4153540	1	427.0288784	511.4738408	-652.0865987	1506.144355
P22105	0.4170430	1	1135.48295	1365.05957	-1744.540996	4015.506896
P00918	0.4195400	1	-373.0446016	450.929457	-1324.422595	578.3333914
P08294	0.4201650	1	-401.0630634	485.4618637	-1425.298066	623.171939
Q6UXB8	0.4227120	1	-11949.72684	14545.54907	-42638.15286	18738.69918
P01011	0.4256030	1	-26946.93825	33009.82926	-96591.59025	42697.71374
Q03591	0.4283050	1	-3379.449249	4164.536024	-12165.85223	5406.95373
P48740	0.4303970	1	-1045.457372	1294.297086	-3776.185527	1685.270782
O75636	0.4309050	1	-2088.362924	2588.335466	-7549.273411	3372.547564
Q8NCS4	0.4330920	1	178.8089663	222.6926863	-291.0315323	648.6494648
Q9Y2I7	0.4339960	1	-401.7107042	501.301683	-1459.364804	655.9433957
Q01082	0.4372140	1	4355.639606	5474.424982	-7194.387502	15905.66671
P22314	0.4387070	1	-423.2098225	533.6837328	-1549.184076	702.7644307
Q92503	0.4391160	1	-506.9125779	639.8172676	-1856.809016	842.9838603
O95294	0.4419910	1	-488.5211551	620.565674	-1797.800281	820.757971
P02743	0.4423420	1	18775.32896	23868.85108	-31583.54487	69134.20279

Q96M86	0.4445010	1	716.1843754	914.8766326	-1214.036596	2646.405347
P02748	0.4522090	1	-4664.986201	6063.078137	-17456.9629	8126.990502
Q96S96	0.4556880	1	140.2162567	183.6727042	-247.299276	527.7317893
P01031	0.4615950	1	13793.00592	18310.90737	-24839.63169	52425.64352
Q96HW7	0.4628790	1	-1161.475411	1546.418421	-4424.133086	2101.182264
P02775	0.4632110	1	-1612.322856	2148.307484	-6144.855453	2920.209741
P0DPA3	0.4682090	1	787.2147184	1060.920617	-1451.132127	3025.561564
P07358	0.4685830	1	-4461.821621	6018.285991	-17159.29516	8235.651916
P36980	0.4738040	1	-4417.855989	6030.67119	-17141.46001	8305.748033
Q5VT06	0.4764400	1	-6338.899504	8705.628107	-24706.1693	12028.37029
Q9NP66	0.4835330	1	-56.9689713	79.5327248	-224.7683529	110.8304104
Q9Y4P3	0.4844190	1	56.1592604	78.563658	-109.5955691	221.9140898
P02774	0.4871950	1	-22627.89425	31860.27961	-89847.20848	44591.41999
P07437	0.4881330	1	-78.992693	111.4658547	-314.1650896	156.1797036
Q0JRZ9	0.4983450	1	-2701.070738	3903.870624	-10937.51779	5535.376318
P43121	0.4991200	1	-6388.688125	9250.529942	-25905.6003	13128.22405
P61769	0.5032690	1	-456.7417303	667.8713728	-1865.827157	952.3436961
P0CF74	0.5037180	1	256.7112289	375.7772081	-536.1093787	1049.531836
P04278	0.5070730	1	-504.4843661	744.3923413	-2075.014924	1066.046192
O43866	0.5083100	1	-1147.64607	1698.418073	-4730.994979	2435.702839
O14556	0.5133250	1	-277.5165369	415.6668153	-1154.496859	599.4637852
P02746	0.5194270	1	-2430.477797	3694.425076	-10225.03337	5364.077779
P07195	0.5253090	1	-40.8247245	62.9500219	-173.6376614	91.9882124

P69905	0.5270880	1	674.7132749	1044.917528	-1529.870002	2879.296552
Q5VUB5	0.5309730	1	473.3847608	740.1538245	-1088.203308	2034.97283
P02790	0.5490610	1	-33962.94266	55554.62377	-151172.9533	83247.068
Q9BXR6	0.5529680	1	-568.692392	939.5035026	-2550.871517	1413.486733
P05090	0.5561470	1	-106.3210453	177.0780356	-479.9230433	267.2809527
Q70CQ4	0.5577840	1	-1460.863077	2443.2931	-6615.76092	3694.034767
Q13474	0.5596600	1	-28.5159085	47.9232741	-129.6251787	72.5933617
P0C0L5	0.5606640	1	-8116.930549	13676.49148	-36971.80533	20737.94423
Q96SN8	0.5608970	1	-435.0421084	733.4563665	-1982.499776	1112.415559
P05362	0.5627050	1	-81.1413433	137.4401471	-371.1147066	208.83202
Q9Y6R7	0.5681760	1	261.0611825	448.5241223	-685.2419978	1207.364363
P02649	0.5749980	1	-2252.662076	3940.195468	-10565.74786	6060.423703
Q6ZN68	0.5768030	1	-20.590245	36.1872601	-96.93869	55.7582
P07737	0.5788710	1	-778.0914898	1375.01959	-3679.129241	2122.946262
O00391	0.5796920	1	-544.9307471	965.0897308	-2581.092095	1491.230601
Q6IPM2	0.5851710	1	475.0834062	853.814229	-1326.307155	2276.473967
Q9Y2T6	0.5867990	1	144.1366957	260.1787595	-404.7925042	693.0658956
Q9NPY3	0.5878620	1	108.5627193	196.5281177	-306.0753648	523.2008035
Q9ULB1	0.5903720	1	-182.0455155	331.7993074	-882.0808629	517.989832
Q86YA3	0.5936480	1	-161.4629911	296.9225575	-787.9148283	464.9888461
P0DOX7	0.5940530	1	-1081.535011	1991.092031	-5282.371996	3119.301974
P04003	0.5950760	1	11741.87224	21677.25032	-33993.12818	57476.87265
Q9NZP8	0.5957530	1	277.1223698	512.5596747	-804.2840165	1358.528756

P13796	0.5969120	1	968.1045006	1796.293662	-2821.74385	4757.952851
P12830	0.5999050	1	-96.598456	180.7206955	-477.8857946	284.6888827
P68104	0.5999430	1	-135.6735408	253.8510477	-671.2524356	399.905354
P03951	0.6006940	1	-426.9669909	800.5344598	-2115.947065	1262.013083
P01034	0.6007960	1	-88.8662259	166.6653584	-440.4993952	262.7669435
Q9Y6U3	0.6020320	1	-103.6584512	195.0751213	-515.2309809	307.9140786
P19652	0.6023780	1	117.7398081	221.7874491	-350.1908069	585.6704231
P0DP02	0.6031750	1	-269.497087	508.7806503	-1342.930429	803.9362548
Q13790	0.6055030	1	-71.7458805	136.3302608	-359.3775884	215.8858274
P02765	0.6070990	1	-19621.69911	37451.77857	-98638.04497	59394.64674
P02749	0.6181670	1	-11069.49063	21801.94026	-57067.56383	34928.58256
P23443	0.6234980	1	-42.6128005	85.2286771	-222.4295911	137.2039901
P0DOX5	0.6305540	1	-494.6840071	1010.024299	-2625.649008	1636.280994
Q13617	0.6312770	1	252.3655099	516.3684057	-837.0765963	1341.807616
O75530	0.6357150	1	-184.122961	381.7274845	-989.4975543	621.2516324
Q8IVW1	0.6444380	1	79.674739	169.5774874	-278.1024855	437.4519636
P55899	0.6449000	1	-12731.34796	27135.12151	-69981.45003	44518.75411
P08697	0.6455190	1	2084.814959	4451.881091	-7307.833118	11477.46304
P42702	0.6460130	1	-581.6146968	1243.843632	-3205.895368	2042.665974
P52789	0.6525650	1	120.9010043	263.8222821	-435.7153563	677.5173649
Q9UK55	0.6542320	1	-489.7561757	1074.261797	-2756.25045	1776.738099
Q06828	0.6615970	1	-11328.59524	25430.32706	-64981.89541	42324.70494
Q6ZTR5	0.6652560	1	-96.0772077	218.2009707	-556.4410148	364.2865994

P54108	0.6671860	1	-142.4064212	325.4274914	-828.998412	544.1855697
P00450	0.6682890	1	23780.80208	54537.23545	-91282.70684	138844.311
P20472	0.6685820	1	-6.7981089	15.6050354	-39.7218557	26.1256379
Q9NWQ4	0.6750780	1	58.3520849	136.8084628	-230.2885411	346.9927109
O00750	0.6763800	1	-42.6455905	100.4128937	-254.4982777	169.2070968
P41594	0.6800710	1	-34.8510545	83.06842	-210.1101011	140.4079921
P15169	0.6827560	1	-544.0253506	1308.375287	-3304.455913	2216.405212
P32119	0.6832100	1	-276.4947226	665.979722	-1681.589115	1128.599669
Q8NHU2	0.6861850	1	-90.6409249	220.5214404	-555.9004952	374.6186453
P11226	0.6875550	1	126.4177091	308.9938732	-525.5023781	778.3377962
Q15166	0.6876120	1	-1206.345261	2949.154088	-7428.516498	5015.825976
P22891	0.6925160	1	77.820558	193.4656332	-330.3562488	485.9973648
Q96B67	0.7003680	1	324.2167017	828.3643152	-1423.479235	2071.912638
P00748	0.7035460	1	-1820.192427	4703.14559	-11742.96226	8102.577403
Q8IYX8	0.7040870	1	-272.9956139	706.7451846	-1764.097614	1218.106386
P05154	0.7061750	1	1701.893637	4438.946209	-7663.464224	11067.2515
Q9Y2Q0	0.7069850	1	-214.9963682	562.3944395	-1401.544918	971.5521811
Q9Y6J0	0.7240010	1	388.9251058	1083.299898	-1896.637894	2674.488105
Q5JRA6	0.7285160	1	-21.1823261	60.027097	-147.8284306	105.4637783
P29120	0.7286730	1	-75.0721552	212.8698744	-524.1883323	374.0440218
O75882	0.7299100	1	-630.6498004	1796.787191	-4421.539407	3160.239806
P68363	0.7300570	1	-37.8778533	107.9796503	-265.6950015	189.9392949
P43652	0.7379670	1	-2191.205155	6443.317096	-15785.41594	11403.00563

P06576	0.7405780	1	-1783.553797	5299.579045	-12964.68822	9397.580628
A6NKP2	0.7420820	1	413.5592462	1236.291901	-2194.788666	3021.907158
P02042	0.7446970	1	4.7939885	14.4837992	-25.7641566	35.3521337
Q9UHF7	0.7482710	1	156.8634357	480.9143055	-857.7770576	1171.503929
Q6ZMB0	0.7500470	1	86.6809812	267.708407	-478.1343862	651.4963485
P05546	0.7516510	1	-2684.328402	8345.906517	-20292.65198	14923.99518
B9A064	0.7552700	1	443.1774091	1399.020796	-2508.49846	3394.853278
Q9NQ76	0.7572460	1	60.5044401	192.6106177	-345.8684417	466.8773218
Q8TE12	0.7637780	1	-960.2267423	3144.280757	-7594.079265	5673.62578
Q15195	0.7684670	1	-290.3599317	970.6799271	-2338.315563	1757.5957
P05160	0.7732140	1	-1536.63458	5247.94224	-12608.82487	9535.555709
P58397	0.7746950	1	684.5186213	2353.626122	-4281.198435	5650.235678
P35858	0.7747470	1	-1219.106595	4192.735847	-10065.006	7626.792808
P18428	0.7822940	1	-165.8052865	590.5894664	-1411.840143	1080.22957
P01861	0.7885950	1	-1022.079928	3752.126488	-8938.374843	6894.214987
P62937	0.7897220	1	34.5896842	127.6794403	-234.7903878	303.9697562
P63104	0.7919870	1	46.0602407	171.9196449	-316.6585042	408.7789857
O75019	0.7999310	1	62.0266736	240.9386426	-446.3094279	570.3627751
Q12805	0.8019430	1	-354.4096517	1390.995786	-3289.15423	2580.334926
P36955	0.8052970	1	-807.5416782	3225.302253	-7612.334615	5997.251258
P10909	0.8066790	1	-1382.333215	5561.374979	-13115.80878	10351.14235
Q16513	0.8091130	1	-304.0854753	1239.332356	-2918.848187	2310.677236
P27169	0.8140250	1	953.9016472	3992.573917	-7469.692998	9377.496292

Q9NVE5	0.8277170	1	-119.5196027	540.7875494	-1260.481599	1021.442393
Q86X10	0.8279760	1	584.2465857	2647.5718	-5001.641642	6170.134813
P68871	0.8331060	1	1282.199752	5992.073072	-11359.96936	13924.36886
Q06033	0.8411120	1	-531.6267505	2611.603409	-6041.628305	4978.374804
B1AKI9	0.8520060	1	-3392.861348	17911.68301	-41183.20918	34397.48648
Q6IA86	0.8557560	1	-43.8724574	237.7101908	-545.397121	457.6522062
A2CJ06	0.8595090	1	-85.2042528	474.1357379	-1085.543219	915.134713
Q96QS6	0.8698830	1	137.6622152	827.7961518	-1608.835001	1884.159432
Q8TDG4	0.8711630	1	32.6478592	198.2893005	-385.7059959	451.0017143
Q9NUB1	0.8720640	1	-125.9605871	770.4682517	-1751.506507	1499.585333
P12814	0.8721730	1	51.1962202	313.423561	-610.0696914	712.4621317
Q86YS3	0.8772790	1	-47.1964408	301.0695274	-682.3976198	588.0047382
Q9P2W1	0.8791570	1	266.7619609	1728.370556	-3379.781162	3913.305083
P06727	0.8812650	1	-1793.566864	11828.67613	-26749.89204	23162.75831
Q15582	0.8816970	1	74.0335152	490.0542118	-959.8904948	1107.957525
P02741	0.8845870	1	-301.3366127	2045.005242	-4615.920528	4013.247303
Q16512	0.8953770	1	7.0562815	52.8619764	-104.4727397	118.5853028
P26927	0.9048360	1	-149.9830352	1235.941216	-2757.591065	2457.624995
P01019	0.9082970	1	-1238.312488	10591.52813	-23584.48352	21107.85855
P22626	0.9134820	1	-62.3421866	565.3298483	-1255.083907	1130.399534
Q15113	0.9166550	1	210.7429029	1984.11042	-3975.364169	4396.849975
P25311	0.9168430	1	-1179.614629	11131.06909	-24664.11759	22304.88833
P20742	0.9171930	1	101.4273834	961.1511437	-1926.424272	2129.279039

P14373	0.9182070	1	-426.5913318	4092.82704	-9061.701577	8208.518914
P50748	0.9225200	1	-24.5877423	249.080831	-550.1023597	500.9268751
P04196	0.9323800	1	-2676.648379	31081.83556	-68253.58923	62900.29247
P38570	0.9356200	1	272.9702728	3329.693256	-6752.068427	7298.008973
P00915	0.9369760	1	-192.1823134	2394.821419	-5244.813849	4860.449222
P04114	0.9370650	1	2530.611421	31579.22576	-64095.73101	69156.95386
Q04727	0.9372020	1	-0.3267696	4.086605	-8.9487524	8.2952133
P20851	0.9401210	1	208.2175921	2731.212701	-5554.137511	5970.572695
A6NHR8	0.9490670	1	123.8442504	1910.349929	-3906.641789	4154.33029
Q9NUJ3	0.9539650	1	11.2843565	192.6120636	-395.0915757	417.6602887
P29622	0.9546380	1	-55.0615837	953.8027061	-2067.409391	1957.286224
Q96KN2	0.9599000	1	-66.0243852	1293.939293	-2795.997663	2663.948892
Q8NEP9	0.9602930	1	-111.4469722	2205.802301	-4765.283029	4542.389084
P00734	0.9617110	1	-844.8835144	17342.1098	-37433.53692	35743.76989
Q5VU43	0.9635230	1	-320.6431037	6908.6444	-14896.60868	14255.32247
Q8WXW3	0.9636520	1	9.013674	194.9036503	-402.1970836	420.2244316
P0C0L4	0.9656800	1	-698.8603312	16004.99204	-34466.44187	33068.72121
P41222	0.9677910	1	-34.7789875	848.717485	-1825.416358	1755.858384
P19320	0.9738350	1	23.6846418	711.5688913	-1477.59449	1524.963773
P01834	0.9745990	1	318.5338812	9857.727437	-20479.45303	21116.52079
P04275	0.9777900	1	120.5722079	4267.62267	-8883.32458	9124.468996
P02750	0.9784280	1	115.3176895	4202.461074	-8751.100149	8981.735528
Q04756	0.9790760	1	19.6413821	737.9393926	-1537.274644	1576.557408

Q96PD5	0.9850680	1	-201.1167082	10589.26066	-22542.50381	22140.27039
Q96EY7	0.9874020	1	55.957452	3492.215539	-7311.973294	7423.888198
Q96JB1	0.9882880	1	-12.5912611	845.2421227	-1795.896259	1770.713736
P17661	0.9895030	1	-8.5192284	638.0974885	-1354.78725	1337.748793
P00747	0.9903900	1	-281.4033778	23021.5798	-48852.69107	48289.88431
Q00059	0.9910310	1	-10.6751027	935.8313009	-1985.10656	1963.756354
O95045	0.99267	1	-0.8730756	93.6503137	-198.4579663	196.7118152
P04070	0.993344	1	-4.2277446	499.3668381	-1057.799679	1049.34419
P19838	0.997508	1	-0.4119934	129.9936774	-274.674679	273.8506922

Declaração de direitos autorais

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam na minha dissertação de mestrado intitulada “Compreensão da depressão geriátrica através de biologia de sistemas a partir da análise proteômica de plasma sanguíneo”, não infringem os dispositivos da lei nº 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 15 de junho de 2020

Autora: Licia Carla da Silva Costa
RG: 10.372.894-5 – SSP/SP

Orientador: Daniel Martins de Souza
RG: 32.431.379-2 – SSP/SP

Bioética – Biossegurança

UNIVERSIDADE FEDERAL DE MINAS GERAIS

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Depressão geriátrica, sobrecarga alostática e declínio cognitivo
Pesquisador: Breno Satler de Oliveira Diniz
Área Temática:
Versão: 3
CAAE: 20204313.2.0000.5149
Instituição Proponente: Faculdade de Medicina da UFMG
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.458.177

Apresentação do Projeto:
Emenda para inclusão de equipe do projeto.

Estudo de coorte longitudinal com os pacientes e idosos controles provenientes da comunidade e avaliados no serviço de Geriatria do HC-UFMG (Instituto Jenny Andrade de Farias) para avaliação clínica e psiquiátrica, coleta de material biológico e avaliação de marcadores biológicos.

Objetivo da Pesquisa:
Em PB_INFORMAÇÕES_BÁSICAS_1388649_E1.pdf os objetivos são descritos:
Objetivo Primário: Desenvolver um índice de toxicidade sistêmica, em idosos com depressão, a partir da análise integrada de marcadores biológicos relacionados a inflamação, estresse oxidativo e suporte neurotrófico.
Objetivo Secundário: Avaliar a relação entre o índice de toxicidade sistêmica e: - Parâmetros de gravidade do episódio depressivo: gravidade de sintomas depressivos, déficits cognitivos, comorbidades clínicas. - Evolução do quadro depressivo: recorrência de episódios depressivos, declínio cognitivo, pior resposta ao tratamento.

Avaliação dos Riscos e Benefícios:
Segundo PB_INFORMAÇÕES_BÁSICAS_1388649_E1.pdf:
Riscos: Os riscos associados a este projeto são mínimos e relacionados a complicações da punção venosa em veia antecubital (dor local leve, hematoma local) e o desconforto com relação a

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entrevista psiquiátrica e neuropsicológica. Estes procedimento serão realizados por profissionais de saúde treinados para a sua realização.

Benefícios: O principal benefício direto em potencial da participação é a possibilidade de receber o tratamento para quadro depressivo, o acompanhamento psiquiátrico para avaliação de declínio cognitivo e a possibilidade de se realizar intervenções já referendadas pela literatura caos o paciente apresente recorrência de episódio depressivo e o diagnóstico de quadros demenciais no acompanhamento longitudinal. Além disto, o conhecimento mais detalhado sobre os mecanismos subjacentes da relação entre quadros depressivos e a síndrome metabólica e os impactos deste quadro sobre sintomas depressivos e déficits cognitivos podem auxiliar no estabelecimento de novas estratégias de prevenção e tratamento para os quadros depressivos nestes pacientes.

Comentários e Considerações sobre a Pesquisa:

A hipótese da pesquisa é descrita em PB_INFORMAÇÕES_BÁSICAS_1388649_E1.pdf: "Idosos portadores de quadros depressivos apresentam um conjunto de alterações em marcadores biológicos relacionados a maior status próinflamatório, redução do suporte neurotrófico e maior estresse oxidativo. Este cojunto de alterações podem ser agrupados em um Indice de Toxicidade Sistêmica e a identificação deste conjunto de alterações podem auxiliar na identificação de idosos sob maior risco de declínio cognitivo e outros desfechos negativos de saúde."

Considerações sobre os Termos de apresentação obrigatória:

Segundo PB_INFORMAÇÕES_BÁSICAS_1388649_E1.pdf: Justificativa da Emenda: "O projeto 20204313.2.0000.5149 obteve sua aprovação junto ao CEP UFMG na data de 20/11/2013, número do parecer 452.219. O projeto encontra-se em desenvolvimento. Esta emenda tem como objetivo incluir dois pesquisadores na equipe de pesquisa: Licia Carla da Silva Costa e Dr. Daniel Martins de Souza, com o propósito de desenvolver classificadores diagnósticos a partir da investigação por biomarcadores.Este objetivo já encontra-se descrito na apresentação do projeto aprovado. A busca por biomarcadores é um subprojeto contido no projeto 20204313.2.0000.5149 já aprovado, e é tema do mestrado de Licia Carla da Silva Costa, sob o título "Identificação de biomarcadores em depressão geriátrica através de análise proteômica", e, sendo assim, no parecer, é necessário que essas informações (nome de cada membro da equipe e do projeto de mestrado) estejam contidas, por exigência da pós-graduação."

Conclusões ou Pendências e Lista de Inadequações:

Emenda aprovada para inclusão de pesquisador.

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Continuação do Parecer: 3.458.177

Considerações Finais a critério do CEP:

Tendo em vista a legislação vigente (Resolução CNS 466/12), o CEP-UFMG recomenda aos Pesquisadores: comunicar toda e qualquer alteração do projeto e do termo de consentimento via emenda na Plataforma Brasil, informar imediatamente qualquer evento adverso ocorrido durante o desenvolvimento da pesquisa (via documental encaminhada em papel), apresentar na forma de notificação relatórios parciais do andamento do mesmo a cada 06 (seis) meses e ao término da pesquisa encaminhar a este Comitê um sumário dos resultados do projeto (relatório final).

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1388649_E1.pdf	28/06/2019 16:06:27		Aceito
Outros	Escala de Ansiedade Generalizada.docx	05/11/2013 16:09:23		Aceito
Outros	CIRSG manual.doc	05/11/2013 16:08:55		Aceito
Outros	PHQ9.docx	05/11/2013 16:08:44		Aceito
Outros	HDRS-21.docx	05/11/2013 16:08:31		Aceito
Outros	MINI5_PROTOCOLO.doc	05/11/2013 16:08:15		Aceito
Outros	Parecer do COEP.docx	05/11/2013 16:07:44		Aceito
Outros	Parecer_INCT.pdf	05/11/2013 16:07:29		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_Indice de Toxicidade Sistemica.doc	05/11/2013 16:07:08		Aceito
Projeto Detalhado / Brochura Investigador	Roteiro para Cadastro de Pesquisa_CNPq_Universal_2013.doc	05/11/2013 16:06:55		Aceito
Outros	Parecer do Departamento.pdf	08/10/2013 15:27:49		Aceito
Outros	depe.pdf	08/10/2013 15:27:14		Aceito
Outros	Aprovacao_unidade_funcional.pdf	12/08/2013 16:02:23		Aceito
Folha de Rosto	Folha_de_rostro.pdf	12/08/2013 16:01:50		Aceito

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Continuação do Parecer: 3.458.177

Outros	termo compromisso (2).jpg	24/07/2013 18:32:06		Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BELO HORIZONTE, 17 de Julho de 2019

Assinado por:
Críssia Carem Paiva Fontainha
(Coordenador(a))

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